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Menadione Resistance:
A Model For
Cellular Defences Against Oxidative Stress

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ABSTRACTS AND PRESENTATIONS ARISING FROM RESEARCH

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ABBREVIATIONS

A	adenine
ADP	adenosine diphosphate
α-MEM	alpha-minimal essential medium
AMP	adenosine monophosphate
AP-1	activator protein-1
APS	ammonium persulphate
ARE	antioxidant responsive element
ATP	adenosine triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
BSO	buthionine-S,R-sulphoximine
C	cytosine
°C	Celsius (degree)
Ca^{2+}	calcium
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CHO	Chinese hamster ovary
CuOOH	cumene hydroperoxide
Cu/Zn-SOD	copper/zinc-containing superoxide dismutase
DEM	diethylmaleate
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EMS	ethyl methanesulphonate
ESR	electron spin resonance
FAD	flavin adenine dinucleotide
FID	free induction decay
5FU	5-fluoruracil
G	guanine
GAPDH	glyceraldehyde phosphate dehydrogenase
γ-GCS	gamma-glutamylcysteine synthetase
γ-GT	gamma-glutamyl transferase

GR	glutathione reductase
GRP	glucose regulated protein
GSH	reduced glutathione
GSHPx	glutathione peroxidase
GSSG	oxidised glutathione
GST	glutathione S-transferase
Gy	Gray
HBS	hepes buffered saline
HIV	human immunodeficiency virus
HO	heme oxygenase
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HSF	heat shock transcription factor
Hsp	heat shock protein
IEF	isoelectric focusing
IgG	immunoglobulin G
K	potassium
kb	kilobase
kDa	kilodalton
LDH	lactate dehydrogenase
LTR	long terminal repeat
M	molar
mBBr	monobromobimane
Mn-SOD	manganese-containing superoxide dismutase
MOPS	morpholine propane sulphonic acid
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NAC	*N*-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	*N*-ethylmaleimide
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
$O_2^{-}\cdot$	superoxide anion radical
$\cdot OH$	hydroxyl radical
ORP	oxygen regulated protein

OTZ	2-oxo-thiazolidine-4-carboxylate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDTC	pyrrolidine dithiocarbamate
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
QAO	quinone acceptor oxidoreductase
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
RPMI	Roswell Park Memorial Institute 1640
SDS	sodium dodecyl sulphate
sGSHPx	selenium-dependent glutathione peroxidase
SOD	superoxide dismutase
SSC	150mM sodium chloride and 15mM sodium citrate
T	thymine
TBE	tris boric acid + EDTA
TBST	tris buffered saline tween
TE	tris-EDTA
TEMED	N,N,N',N´-tetramethylene diamine
TK	thymidine kinase
TNF	tumour necrosis factor
tGSHPx	total glutathione peroxidase
TLC	thin-layer chromatography
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRIS	tris(hydroxymethyl)aminoethane
U	uracil
UV	ultraviolet
UVA	ultraviolet A radiation (320-380 nm)
XIP	X-ray-induced polypeptide

ABSTRACT

The abundance of molecular oxygen in the Earth's atmosphere has led to strong evolutionary pressure on organisms to adopt an aerobic existence. The utilisation of oxygen for respiration involves its conversion to water in four sequential electron transfers. At each step, potentially damaging reactive oxygen species are formed. These are detoxified by a system of enzymatic and non-enzymatic antioxidant defence mechanisms. A state of oxidative stress exists when cells either overproduce reactive oxygen species or are deficient in their ability to destroy them. Oxygen radicals are able to interact with all classes of organic molecule. The biological consequences of these interactions are protean but include mutations, cytotoxicity and carcinogenesis. Ionising radiation and some drugs used in the treatment of cancer damage cells by producing oxygen radicals. Therefore, the mechanisms by which cells protect themselves from oxidative stress are likely to be partly responsible for the clinical phenomena of chemo- and radioresistance.

The response of prokaryotes to oxidative stress has been thoroughly investigated. Bacteria protect themselves from the lethal effects of oxidants by inducing the expression of protective stress genes. The pattern of induction depends on the type of the reactive oxygen species to which the cell is exposed. An adaptive response, in which exposure to one episode of oxidative stress leads to protection from a second, is observed in bacteria. The eukaryotic response to oxidative stress is less well characterised but certain genes that are specifically induced by oxidants have now been identified.

To study the genetic changes which confer resistance to oxidants, cells lines that are resistant to the redox-cycling agent, menadione, have been isolated from Chinese hamster ovary (CHO) and human transitional carcinoma (EJ-WT) parental cell lines. They exhibit cross-resistance to chemical oxidants (hydrogen peroxide and sodium arsenite) but not to ionising radiation (in oxic conditions). The concentrations of the major sources of intracellular thiol groups, glutathione and cysteine, are two-fold greater in menadione-resistant than in the corresponding parental cell lines. Exposure to menadione results in depletion of both glutathione and cysteine but the subsequent recovery of thiols is more rapid and of greater magnitude in menadione-resistant than sensitive cell lines. ^{1}H spin echo nuclear magnetic resonance (NMR) spectroscopy was used to study intact cells. Using this technique the removal of menadione from suspensions of resistant and sensitive cells was observed. However, only in menadione-sensitive cells was concomitant depletion of the NMR-visible pool of glutathione observed.

The acquisition of resistance to menadione was associated with significant changes in the expression of several enzymes that are implicated in the oxygen-induced stress response and in protection from redox-cycling agents. The transcription of genes encoding heme oxygenase and the glutathione-related enzymes, GST-Pi and glutathione peroxidase, increases in CHO parental cells after transient oxidative stress. These genes are constitutively induced in CHO menadione-resistant cell lines. This suggests that resistance results from perpetuation of a response that normally occurs only transiently. The eukaryotic transcription factor, NF-kB, activates the expression of many genes involved in inflammatory and immune responses. Diverse agents induce NF-kB DNA-binding and there is evidence that they do so through the mediation of oxyradicals. However, the increased concentration of low molecular weight thiols in menadione-resistant CHO cells was not associated with a change in the ability to activate NF-kB following oxidative stress.

Menadione resistant cell lines provide a useful model for the study of molecular mechanisms involved in defence against oxidative stress. The observed changes in antioxidant defences that occur synchronously with the acquisition of menadione-resistance constitute an adaptive response. Such an adaptive response, if elicited *in vivo*, would result in resistance of tumours to certain chemotherapeutic agents.

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW OF CELLULAR STRESS RESPONSES

In order to survive organisms must be able to adapt to a continuously changing environment. They are exposed to endless variation in, for example, ambient temperature, intensity of ultraviolet light and ionising radiation as well as in the chemical content of both their foodstuffs and the atmosphere in which they live. An ability to adapt to such change rapidly and appropriately may make the difference between survival and extinction. Adjustment to fluctuations in the environment takes place on many levels. There may be major physiological adaptations to change. Warm-blooded creatures, for example, respond to a fall in temperature by shivering which generates heat and by constriction of the cutaneous vasculature which minimises heat loss through the skin. Response to fluctuations in environmental conditions also takes place at cellular and molecular levels. Both eukaryotic and prokaryotic cells deal with many types of environmental challenge by activating co-regulated groups of genes that affect a variety of cellular functions (Gottesman, 1984). The genes within each group and the proteins that they encode constitute a stimulon. The synthesis of proteins other than those required for protection from the immediate crisis may be suppressed. An adaptive response, in which exposure to one episode of stress leads to protection from a second, is seen for some types of cellular insult. When an environmental challenge is long-lasting, the molecular response that it provokes may become immortalised.

1.1.1 Induction of stress responses

Bacteria are able to respond with specific homeostatic responses when confronted by adverse environmental change. In some cases analogous responses have been identified in eukaryotic cells. The response of cells to heat shock has been the most well characterised of all the stress responses. It is a rapid and highly conserved response to elevated temperature. The genes encoding the heat shock proteins (hsps) are activated within minutes of the shift in temperature. There is simultaneous transcriptional inhibition of normal protein synthesis (Ashburner, 1982). The collective function of the hsps appears to be protection of the cell during

the period of elevated temperature and, subsequently, restoration of the metabolic pathways that were perturbed. The heat shock response was first described in 1962 when Ritossa reported that, following a shift in temperature from 20° to 37°C, several new puffs appeared in the salivary gland polytene chromosomes of *Drosophila melanogaster* (Ritossa, 1962). Over the following decade it became clear that these puffs were the sites of vigorous RNA transcription and, in some cases, translation into hsps. The hsps represent only 2 to 3% of total cellular proteins in unstressed cells but, following exposure to heat, they may account for as much as 20% of cellular protein. They have been classified into four major families according to their molecular weight: 83-90kDa, 66-78kDa (hsp70), 60kDa (hsp60) and 15-30kDa (the small heat shock proteins). There is remarkable conservation of the heat shock genes and hsps across species. There is, for example, 50% homology in the 70kDa hsp in *E. coli* and humans. The functions of the hsps include binding of polypeptides and facilitating their transport to their correct intracellular destination, transporting proteins across membranes and preventing proteins from misfolding. All of these functions depend on the ability of hsps to recognise unstructured regions in other proteins.

The hsps are under the control of transcription factors, including σ^{32}, which itself is encoded by the *rpoH* gene. A shift to high temperature is followed by *rpoH* mRNA stabilisation and stimulation of *rpoH* gene transcription. Thus, σ^{32} accumulates and promotes transcription of the hsp-encoding genes. The transcription of *rpoH* is itself controlled by a number of factors including σ^{24}, which seems to be specifically active at temperatures above 50°C (Wang and Kaguni, 1989). The heat shock response of eukaryotes has also been intensively studied. All eukaryotic genes that are transcriptionally induced following exposure to heat shock contain a sequence motif located in the 5'-flanking region referred to as the heat shock element. This binds the heat shock transcription factor (HSF) which has a role analogous to that of σ^{32} in bacteria. The cellular response to heat is clearly complex but the involvement of several transcription factors and the potential for control of the response at different levels allows "fine-tuning" to take place. This co-ordinated regulation of many genes is typical of the other stress responses.

Although heat shock has been most intensively studied, specific responses have also been observed following many different types of environmental challenge and they too are defined by induction of unique sets of genes. Starvation (Jenkins, 1988), cold shock (Jones *et al*, 1987), thiols (Javor, 1989), detergents (Adamowicz *et al*, 1991), change in pH (Taglicht *et al*, 1987; Hickey and Hirshfield, 1990), anaerobiosis (Smith and Neidhardt, 1983), DNA damage (Demple and Halbrook, 1983; Farr *et al*,

1985), ionising radiation (Boothman *et al*, 1990) and oxidants (Imlay and Linn, 1987; Greenberg *et al*, 1990) can all induce characteristic groups of proteins. The physiological relevance of these responses is not always obvious. Why, for example, do bacteria develop a complex response to the presence of detergents? *E. coli* respond to treatment with detergents by the induction of nineteen proteins as well as by the depression or complete cessation of synthesis of twenty eight others. It is postulated that they do so because enteric bacteria are likely to encounter bile salts (natural detergents) in the gastrointestinal tracts of their animal hosts.

Exposure of bacteria to thiols and, therefore, to a reducing environment, leads to the stimulation of 18% and inhibition of the synthesis of 44% of all cellular proteins within 30 minutes. It has been postulated that these changes represent a reductive stress regulon. Cells also respond to UV radiation and other DNA damaging agents by the induction of a particular set of proteins. This is known as the SOS response (Walker, 1984). Characterisation of the SOS response has revealed many DNA repair functions, including genes involved in DNA excision (*uvr*), recombinational repair (*recA*) and site-specific recombination (*himA*). Boothman *et al* (1989) identified a protein expression response that is activated by ionising radiation. Eight major X-ray-induced polypeptides (XIPs) were identified when plateau phase human malignant melanoma cells were irradiated. The expression of XIPs appeared to be specific for ionising radiation damage since heat shock, hypoxia and alkylating agents failed to induce their synthesis.

A reduction in temperature from 37°C to 10°C in *E. coli* results in marked slowing of cell growth. Although the synthesis of most proteins is dramatically reduced a small group of thirteen proteins is induced. This is thought to constitute a so-called "cold shock response" which is analogous to the heat shock response. Several cold shock proteins have been identified and are involved in transcription and translation. They include polynucleotide phosphorylase which degrades single-stranded RNA *in vitro* in the 3'-to-5' direction and *RecA* which is involved in recombination and in the induction of the SOS response needed for DNA repair (Jones *et al*, 1987).

E. coli are capable of growth in both aerobic and anaerobic conditions. Eighteen polypeptides are produced in increased amounts by *E. coli* growing anaerobically and five have been identified (Smith and Neidhardt, 1983). These five enzymes are all involved in fermentation, the process by which bacteria derive energy during anaerobic growth. They are four glycolytic enzymes and pyruvate formate-lyase. Glucose-starvation of *E. coli* results in the induction of a specific set of about thirty proteins (Groat *et al*, 1986).

E. coli treated with non-toxic levels of the superoxide-generating redox-cycling agents, menadione (2-methyl-1,4-naphthoquinone) and paraquat (1,1'-dimethyl-4,4'-bipyridium dichloride) show dramatic changes in protein composition with the induction of 40 or so proteins. A specific response to H_2O_2 is also observed in *E. coli* and consists of the induction of approximately 35 proteins. The responses to oxidative stress are dealt with in more detail in section 1.2.

1.1.2 Overlap of stress responses

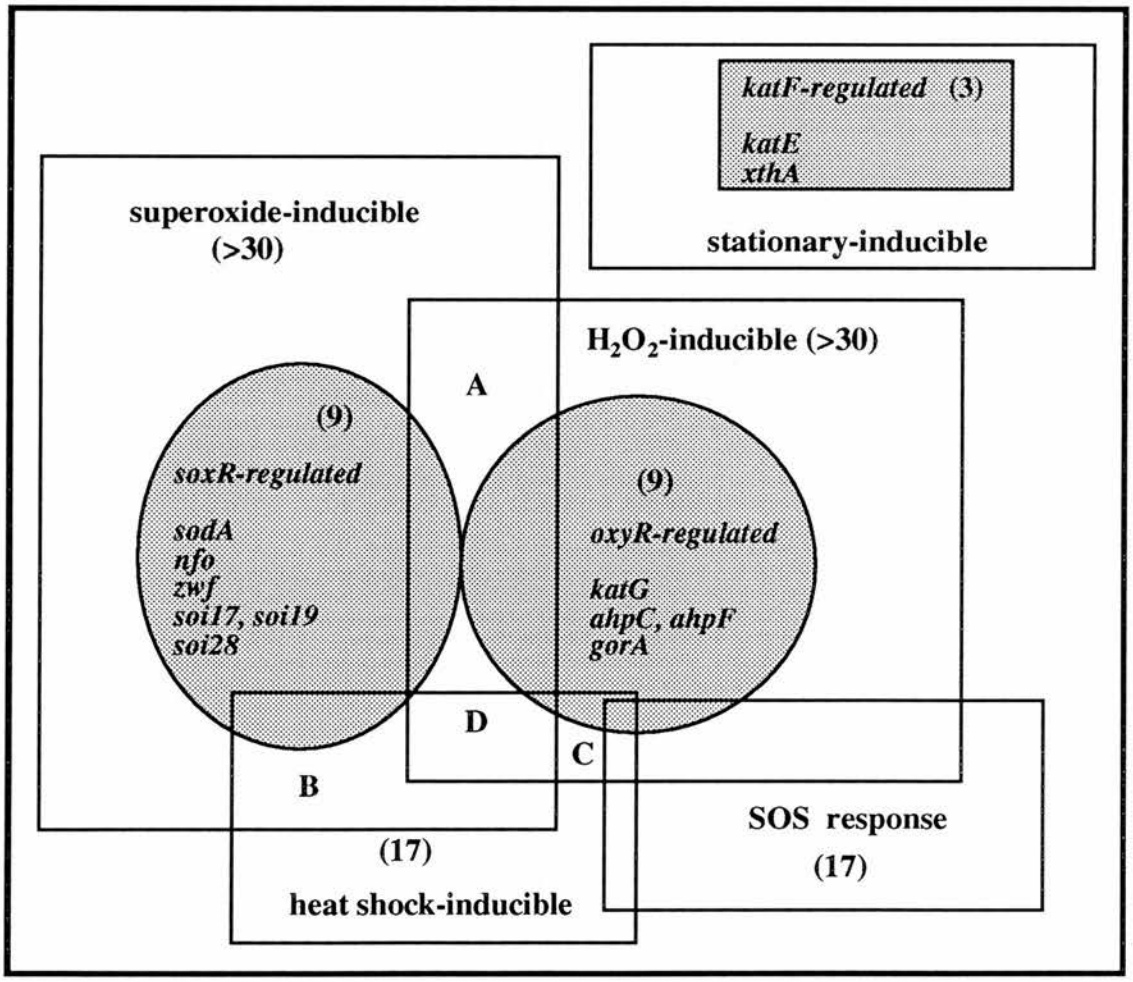
Proteins that belong to one stimulon may also be inducible by other stresses. For example, treatment of cells with the oxidant, H_2O_2, induces a number of proteins that also form part of the heat shock response. This is true of both bacterial (Morgan, 1986) and mammalian cells (Burdon *et al*, 1987). It has been shown that oxygen free radicals induce some hsps and this may explain the apparent overlap between the two responses. Since it damages DNA, H_2O_2 also induces the SOS response. A crossover of induction of stress proteins by both hydrogen peroxide and UVA radiation has been observed in bacteria as well as in mammalian cells (Keyse and Tyrrell, 1990). The GroE and DnaK heat shock proteins are induced following treatment with H_2O_2, paraquat and nalidixic acid. They therefore form part of at least four overlapping responses and may serve as general antistress proteins.

Another example of overlap of different stress responses is the response of *E. coli* to treatment with thiols that is, paradoxically, associated with transient stimulation of some oxidative-stress genes such as *sodB* and *ahp* which encode superoxide dismutase and alkyl hydroperoxide reductase respectively. This may occur because thiols are able to generate low levels of hydrogen peroxide (Ehrenberg *et al*, 1974). Treatment with thiols also induces a slight and transitory increase of *rpoH* and other heat shock genes. Similarly, thirty proteins are induced following glucose-starvation of *E. coli* and subsets of these are also synthesised following heat- and H_2O_2-treatment (Jenkins *et al*, 1988). There are, for example, eleven proteins common to starvation and heat shock and three proteins common to all three responses. Three of the heat shock proteins (DnaK, GroEL and HtpG) are regulated by σ^{32}, which has been shown to increase on starvation (Jenkins *et al*, 1991).

There is then considerable overlap of the sets of proteins induced by noxious insults. Overlap of the stimulons involved in oxidative stress is illustrated in Figure 1.1.

Figure 1.1 *Oxidative stress-inducible genes in bacteria and possible overlaps with other stress responses*

Each box represents a stimulon and the shaded areas contain the names of co-regulated groups of genes (regulons). (A) Genes induced by both superoxide-generators and H_2O_2 include *soi-28*, *soi-17/19* and *recA*, (B) genes induced by both superoxide and heat include *sodA*, *nfo* and *zwf* which encode superoxide dismutase, endonuclease IV and glucose-6-phosphate dehydrogenase, (C) genes induced by heat and H_2O_2 include *ahpB*, *ahpC* and *dnaK* which encode alkyl hydroperoxides B and C and DnaK, (D) genes inducible by heat, H_2O_2 and superoxide stresses include *groES* and *groEL*. The stationary-inducible genes are not induced by other stresses and so are represented by a separate box. The numbers in brackets indicate the number of proteins and genes known to be involved in the response.



Based on Farr, S.B. (1991). Microbiol. Rev. **55**, 561-585.

1.1.3 Adaptive responses

The treatment of cultured mammalian cells with a variety of environmental stresses induces transient protection against subsequent exposures to the same agent and, sometimes, other agents (Li and Hahn, 1978). The Inducibility of transient thermal resistance by heat shock is well known and occurs in both prokaryotes and eukaryotes. It has been shown that when protein synthesis is inhibited by cycloheximide then induction of thermotolerance does not occur. Thus, the inducible proteins appear to be directly responsible for the increase in thermoresistance (Carper *et al*, 1987). Similarly, following exposure of bacteria to non-lethal H_2O_2 treatments they exhibit enhanced resistance to H_2O_2 and other noxious agents. This adaptive response to H_2O_2 was first reported by Hassan and Fridovich (1977) and Demple and Halbrook (1983) also reported that after a sublethal dose of H_2O_2, cells resist killing by a subsequent dose of peroxide. A similar observation was made in *Bacillus subtilis* by Bol and Yasbin (1990). Christman *et al* (1985) studied the survival kinetics of bacteria after exposure to H_2O_2 and heat. Bacteria became resistant to H_2O_2, other oxidising agents and heat shock following pretreatment with H_2O_2. This adaptation was accompanied by the enhanced synthesis of a set of proteins, five of which were also induced by heat shock. A similar phenomenon has been observed in mammalian cells. In Chinese hamster fibroblasts, Spitz *et al* (1987) reported resistance to H_2O_2 after an initial exposure to either heat or a low concentration of H_2O_2. The timing of the response was the same whichever inducing agent was used. However the magnitude of the induced resistance was greater when H_2O_2 was used as the inducing agent. Both inducing agents also resulted in thermotolerance although the degree of thermotolerance was only modest following pretreatment with H_2O_2 and in this case the kinetics of the response differed according to whether H_2O_2 or heat was used as the adaptive treatment. Thermotolerance was greatest at 6 to 12 hours after heat but maximal at 16 to 36 hours when peroxide was used as the inducing agent.

An adaptive response is also seen in bacteria in response to treatment with superoxide-generating compounds (Farr *et al*, 1985). Pretreatment of bacteria with 1.45mM menadione, a redox-cycling agent, conferred substantial resistance to transient challenge with 100mM menadione (Greenberg and Demple, 1989). Adaptive responses are also seen in yeast. *Saccharomyces cerevisiae* cells treated with low concentrations of H_2O_2 or menadione exhibit adaptive responses (Jamieson, 1992). Pretreatment with menadione is protective against cell killing by hydrogen peroxide. However, pretreatment with H_2O_2 does not protect cells from subsequent

menadione. Heat shock induces resistance to both oxidants but treatment with either oxidant does not protect cells from subsequent menadione.

There is also evidence for inducible cellular resistance to UV radiation in human cells. Arrested populations of human cells when irradiated with low doses of UV and maintained in an arrested state develop enhanced resistance to inactivation by a second dose of UV irradiation (Tyrrell, 1984). An adaptive response following ionising radiation has not been reported although human lymphocytes irradiated with very low doses of X-rays sustain fewer chromosome breaks following a second exposure compared with untreated cells (Youngblom *et al*, 1989). This response has been attributed to induction of repair proteins, because it can be abolished by cycloheximide.

1.2 OVERVIEW OF OXIDATIVE STRESS

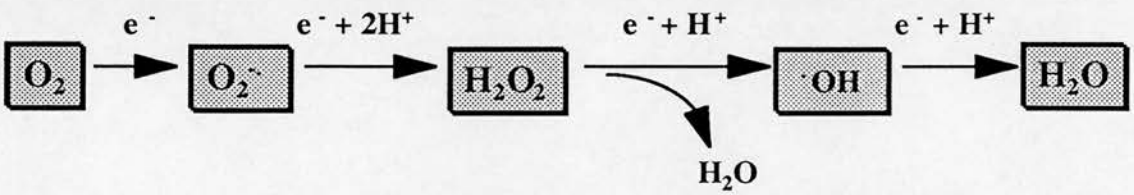
1.2.1 Oxygen toxicity

Aerobic organisms utilise oxygen as the terminal electron acceptor in oxidative phosphorylation. To do so has drawbacks as well as benefits. The advantage of this means of energy generation is that oxygen is in plentiful supply, making up as it does 21% of the earth's atmosphere. The disadvantage is that the reduction of oxygen which takes place as a series of four single electron transfers is accompanied by the generation, at each stage, of reactive oxygen species. Even primitive organisms have numerous and often complex defence mechanisms to detoxify oxygen intermediates as they are formed. Bacteria also require these defences so as to withstand the burst of free radicals to which they are subjected on phagocytosis by macrophages. In normal circumstances homeostasis is maintained and the deleterious by-products of aerobic respiration are dealt with as rapidly as they are produced. If however, active oxygen species accumulate in the cell, either because of unfavourable environmental conditions or because the cell's own defence systems are defective, then a state of oxidative stress is said to exist. An excess of free radicals can wreak havoc with many biochemical processes since they are capable of interacting with all forms of organic molecule.

The reactive oxygen species formed as a result of aerobic metabolism, listed in order of sequential reduction, are the superoxide anion radical ($O_2^{-\cdot}$) and its conjugate acid, the hydroperoxy radical, ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical, ($^{\cdot}OH$) (Fridovich, 1978) (Figure 1.2). Perferyl iron (Fe_3O_2), singlet oxygen ($^1O_2$) and organic oxyradicals are products of reactions involving $O_2^{-\cdot}$, H_2O_2

and ·OH. Singlet oxygen, although highly reactive, is not a free radical. The peroxyl radical, ROO·, is long-lived and so has a diffusion path length allowing it to take part in reactions distant from the site at which it was generated. The reactive oxygen species generated during oxidative stress are listed in Table 1.1.

Figure 1.2 *The univalent pathway of oxygen reduction*



Adapted from Fridovich (1978) Science **201**, 875-880.

The paramagnetic nature of oxygen is the reason that its reduction is accompanied by the generation of free radicals. The outer orbitals of the oxygen atom are occupied by unpaired electrons. These are in parallel spin and to avoid free radical production must each receive simultaneously an electron of complementary spin. If left to chance such an event is rare. However, oxidative enzymes have evolved that circumvent the spin restriction and accomplish the divalent and even tetrameric reduction of oxygen without the release of reactive intermediates. Most of the oxygen consumed by respiring cells is utilised by cytochrome oxidase which reduces oxygen to water without releasing H_2O_2 or $O_2^{-}\cdot$.

Superoxide is generated during normal respiration and cellular metabolism. In *Streptococcus faecalis* in which superoxide dismutase activity has been inhibited by a specific antibody, 17% of the oxygen consumption resulted in superoxide production (Britton *et al*, 1978). Polymorphonuclear leukocytes liberate large amounts of $O_2^{-}\cdot$ during the respiratory burst. Superoxide is also generated through the autooxidation of catecholamines and is liberated as haemoglobin and myoglobin are oxidised to methaemoglobin and methmyoglobin. Superoxide anions are themselves relatively unreactive in an aqueous environment. However, the existence of enzymes with the primary function of elimination of superoxide, suggests that $O_2^{-}\cdot$ can be deleterious to the cell. The toxicity of $O_2^{-}\cdot$ is largely due to its conversion by dismutation to H_2O_2 which takes place either spontaneously or is catalysed by superoxide dismutases.

Table 1.1 *Reactive oxygen species in oxidative stress*

Reactive oxygen species		Half life at 37°C
$O_2^{-}\cdot$	*superoxide anion*	dismutation
$HO_2\cdot$	*perhydroxy radical*	
H_2O_2	*hydrogen peroxide*	stable
$\cdot OH$	*hydroxyl radical*	10^{-9} secs
$RO\cdot$	*alkoxy radical*	10^{-6} secs
$ROO\cdot$	*peroxy radical*	7 secs
ROOH	*organic hydroperoxide*	
$^1O_2$	*singlet oxygen*	10^{-6} secs

Hydrogen peroxide is not a radical and, therefore, is relatively stable. The reductive decomposition of H_2O_2 by reduced metals was first described by Fenton (1894) and subsequently by Haber and Weiss (1934). The direct reaction of superoxide with hydrogen peroxide is slow but superoxide can reduce a ferric compound (equation 1):

$$Fe^{3+} + O_2^{-}\cdot \longrightarrow Fe^{2+} + O_2 \qquad (1)$$

The resulting ferrous compound then reduces H_2O_2 with the generation of $\cdot OH$ (equation 2):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + \cdot OH \qquad (2)$$

Copper and cobalt are also capable of taking part in similar reactions but because of its ubiquity and abundance *in vivo,* iron is more commonly utilised. The most compelling evidence for the involvement of the Fenton reaction in the biological

effects of H_2O_2 comes from studies in which mammalian cells in culture have been treated with H_2O_2 in the presence of the membrane permeable iron-chelator, o-phenanthroline. This drug prevents Fenton reactions from occurring and so abolishes generation of ·OH (Mello-Filho and Meneghini, 1985). To maintain the Fenton reaction an electron source must be available to regenerate the reduced metal. Glutathione and ascorbate are examples of reductants that are capable of reducing transition metals. Treatments that enlarge or deplete the pool of reducing equivalents may, therefore, alter the level of Fenton reactions.

The hydroxyl radical is exceptionally reactive and can damage many biological macromolecules which it does through three types of reaction (Halliwell and Gutteridge, 1984). Firstly, it can cause abstraction of hydrogen atoms. Secondly, it can alter a molecular structure by addition of it itself to, for example, a pyridine ring and thirdly it can cause electron transfer from itself to another atom. Much of the toxicity of $O_2^{-}\cdot$ and H_2O_2 is due to their conversion to the extremely reactive ·OH radical.

1.2.2 Damage caused by oxidative stress

(a) DNA damage

A multitude of different forms of DNA damage are inflicted by oxidants. Hydrogen peroxide, for example, is able to release all four bases and to cleave the backbone of DNA (Rhuse and Freese, 1968). Ananthaswamy and Eisentark (1977) used alkaline sucrose determination to show that H_2O_2 causes accumulation of incisions of DNA in wild-type *E. coli*. Hagensee and Moses (1986) and co-workers demonstrated DNA incision after exposure of the cell to H_2O_2 and that this was followed by the reformation of high molecular weight DNA when H_2O_2 was removed. Attack of the sugar moiety of DNA by H_2O_2 leads to sugar fragmentation and strand breaks with 3'-phosphate or 3'-phosphoglycolate termini (Rhuse and Freese, 1968). Mutant strains of *E. coli* that lack exonuclease III or endonuclease IV, and are therefore defective in their ability to excise such termini and in recombinational DNA repair, are very sensitive to H_2O_2.

Exposure of logarithmically growing *E. coli* to H_2O_2 gives rise to two kinetically distinguishable modes of cell-killing (Imlay and Linn, 1987). Mode 1 is pronounced at low concentrations (1 to 3mM) and is caused by DNA damage. The site of injury of mode 2 which occurs at a higher concentration (more than 20mM) is unknown. Imlay *et al* characterised mode 1 killing by studying the sensitivity of different

mutant strains of *E. coli* to low concentrations of H_2O_2. Strains compromised in their ability to repair DNA damage - *xth*, *recA*, *polA* and *nfo* and *xth* double mutants - were all sensitive to killing by low doses of H_2O_2. Also, *ndh* mutants, that lack NADH dehydrogenase (and therefore have abnormally high levels of reducing equivalents) were especially sensitive to oxidation while starved cells (with low levels of reducing equivalents) were resistant to H_2O_2 (Imlay and Linn, 1986). Hydrogen peroxide therefore constitutes a significant stress *in vivo* by generating DNA damaging radicals.

Oxidative attack on the bases of DNA cause 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and ring-saturated products (Imlay and Linn, 1988). Thymine residues in DNA can be hydroxylated to form 5-hydroxymethyluracil or oxidatively degraded to produce thymine glycol or a urea residue (Demple and Linn, 1982). Addition reactions of ·OH with guanosine residues form 8-hydroxydeoxyguanosine. Intermediate organic radicals that form during the propagation step of lipid peroxidation are able to react with DNA. For example, either oxidising fatty acid transfers free radicals to purines (abstracts a hydrogen atom) resulting in decomposition of the purines, or the fatty acid radicals adds to the purine to form a bulky adduct (Vaca *et al*, 1988). Lipid peroxides decompose to non radical, stable end products such as 4-hydroxyalkenals and epoxides and these are able to interact directly with DNA. They either alkylate bases or form interstrand or intrastrand cross links (Summerfield and Tappel, 1983).

Thymine glycols and 8-hydroxydeoxyguanosine residues are two of the most important predominant stable products of oxygen radical attack on DNA. Thymine glycol can block replication by DNA polymerase *in vitro*. Thymine glycol and its decomposition products are readily removed by N-glycosylase activity associated with endonuclease III (Ide *et al*, 1985; Cunningham and Weiss, 1985). Consistent with the finding that thymine residue damage is a significant component of general oxidative DNA damage - the bulk of the increase in mutagenesis observed in OxyR deletion mutants is found to be A.T to G.C transitions. Evidence that mutations are generated as a result of oxidative DNA damage during aerobic growth has come from studies on the frequencies of spontaneous mutagenesis in bacterial strains that are compromised in their ability to detoxify oxidants. Strains carrying mutations in both superoxide dismutase genes have a significantly increased frequency of mutations (Farr *et al*, 1986).

(b) Membrane damage

The effect of free radical damage on membranes is well documented. Lipid peroxidation occurs when reactive oxygen species set in motion a chain reaction wherein free radicals are passed from one macromolecule to another, resulting in extensive damage to cellular structures. Lipid peroxidation entails three steps: initiation, propagation and termination. The chain reaction begins when hydrogen abstraction from an unsaturated fatty acid gives rise to a lipid radical. The lipid radical reacts with molecular oxygen to form a lipid peroxy radical (ROO·). The reaction is perpetuated when the lipid peroxy radical attacks another unsaturated fatty acid and abstracts a hydrogen atom to form a fatty acid hydroperoxide (ROOH). The peroxides break down thermally or in the presence of oxygen or reduced transition metals to form lipid peroxy radicals or lipid alkoxy radicals both of which take part in more rounds of peroxidation. Lipid alkoxy radicals undergo cleavage of C-C bonds generating products that are shorter than the initial fatty acid. This results in increased fluidity of the cell membrane (Mead, 1976). This then leads to loss of integrity of membrane structure and thus impairment of the transport of nutrients, of ATPase activity, motility and prevents maintenance of osmotic imbalance. Lipid peroxidation alters the proton gradient across the cell membrane, so that the intracellular pH falls. In a relatively acidic environment O_2 coverts to the hydroperoxy radical (HOO·) and this causes further oxidative stress.

Oxidative damage to membranes can be assessed by measuring transport of nutrients. Farr *et al* (1988) studied the uptake of labelled metabolites such as guanosine and glucose by *E. coli* strains after treatment with H_2O_2. Cells treated with 5mM H_2O_2 showed a rapid loss of both proton motive force-dependent and -independent transport within 5 minutes reflecting the inhibition of membrane function. When cells were pretreated with 35μM H_2O_2, inhibition of transport recovered more rapidly. Deletion mutant studies showed that this adaptive phenomenon was dependent on the products of the two genes *oxyR* and *katG*. The protein, OxyR, acts as a transcription factor and controls several oxidant-sensitive genes and *katG* encodes the only inducible catalase gene in *E. coli* (see section 1.5.2). It is possible that different oxidants cause different types of membrane damage. This was demonstrated by the work of Farr *et al* (1988) who found that when exogenous catalase was added to cells that had been challenged with H_2O_2, there was rapid recovery of transport function. This was not the case when cells were challenged with plumbagin (a superoxide-generating agent). In addition, although cells pretreated with H_2O_2 showed enhanced recovery of membrane function when

challenged with H_2O_2 they did not do so when challenged with plumbagin. Interestingly, cells pretreated with plumbagin did not show enhanced recovery of transport when challenged with H_2O_2.

(c) Protein damage

Damage to proteins also results from oxidative stress. The thiol groups of cysteine and methionine are particularly vulnerable to oxidative attack. Interaction of oxyradicals and protein leads to conversion of proline and arginine residues to carbonyl derivatives. Oxidative attack of histidyl and prolyl residues converts them to asperginyl and glutamyl derivatives. Oxidatively modified proteins may be recognised as abnormal by cellular proteolytic systems and are therefore sensitive to degradation. In *E. coli* there appear to be specific proteinases that selectively degrade oxidised proteins (Davies and Lin, 1988). The proteins that are particularly susceptible to oxidative attack have $(Fe\text{-}S)_4$ clusters. It appears that metal-binding sites in proteins are especially sensitive to attack by active oxygen species. Examples of proteins that are degraded more rapidly on oxidation *in vitro* are the ribosomal subunit L12, glutamine synthase and dihydroxyacid dehydrase (Levine *et al*, 1981; Brot and Weissbach, 1983; Kuo *et al*, 1987). Repair of protein damage appears to be limited to the reduction of disulphides and methionine sulphoxides. The reduction of both is facilitated by thioredoxin (Gonzales-Porque *et al*, 1970).

1.2.3 Antioxidant defence mechanisms

Both eukaryotes and prokaryotes are invested with a complex array of antioxidant defences to protect them from the damaging effects of oxidants. These antioxidant components function in a highly integrated manner within the cell (Kong and Fanburg, 1992). They are summarised in Table 1.2.

(a) Enzymatic defences

The primary defence against oxidative stress is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction. The $O_2^{-}\cdot$ radical is eliminated by dismutation to H_2O_2, a reaction that occurs either spontaneously or is catalysed by the superoxide dismutases (SOD). Three distinct types of SOD exist. They all catalyse the same reaction. The iron-containing (Fe-SOD) and manganese-containing (Mn-SOD) enzymes are characteristic of prokaryotes and are closely related. The enzyme containing copper and zinc (Cu/Zn-SOD) is characteristic of

Table 1.2. *Antioxidant defences in biological systems*

Non-enzymatic	**Ancillary enzymes**
Vitamin E	QAO
Vitamin C	Conjugation enzymes
GSH	GSSG reductase
Flavanoids (plants)	NADPH supply
β-carotene	Metallothioneins
	Thioredoxin
Enzymatic	**Transport systems**
Superoxide dismutase	GSSG export
GSH peroxidase	Conjugate export
Catalase	
	Transcription factors
Sequestration of metal ions	NF-κB
	Jun/Fos
Lactoferrin	
Transferrin	**Oncogenes**
Caeruloplasmin	
	Bcl-2

eukaryotes and is cytoplasmic in location. It has no sequence homology with the other two types of SOD. Eukaryotes also contain Mn-SOD (located in the mitochondria). The reaction that the SODs catalyse is as follows (E = enzyme, M= metal):

$$E\text{-}Me^{n} + O_2^{-}\cdot \longrightarrow E\text{-}Me^{n-1} + O_2 \qquad (3)$$

$$EMe^{n-1} + O_2^{-}\cdot + 2H^{+} \longrightarrow E\text{-}Me^{n} + H_2O_2 \qquad (4)$$

In Cu/Zn-SOD it is the copper that participates in the catalytic cycle, oscillating from cupric to cuprous forms. That SOD is important in cellular defence against oxidative damage was demonstrated by Carlioz and Touati (1986). They isolated strains of *E. coli* that carried mutations in the genes encoding the superoxide dismutases (*sodA*, *sodB*). Although single mutations did not affect growth, the strains with mutations at both loci were unable to grow on minimal medium under aerobic conditions. The double mutant strains were only viable when the minimal medium was supplemented with amino acids, particularly leucine, isoleucine and valine. These experiments showed that the superoxide dismutases are essential under aerobic, nutrient-limiting conditions and that the synthesis of certain amino acids, particularly the branched chain amino acids, is sensitive to damage by superoxide radicals. There is evidence for the inducibility of SOD activity in bacteria. The redox-cycling agent, paraquat, leads to an elevation of Mn-SOD activity in *E. coli* and these cells are then rendered resistant to the lethal effect of hyperbaric oxygen (Hassan and Fridovich, 1977). The SOD enzymes are only slightly inducible by oxidants in mammalian cells (for further discussion see page 160).

Enzymatic defence against H_2O_2 is provided by catalase and glutathione peroxidase (glutathione peroxidase is discussed in section 1.4.4). Catalase-deficient *E. coli* mutants show only slightly enhanced killing by H_2O_2 which raises the question as to the role played by catalase in protection against H_2O_2 in bacteria (Eisenstark and Perrot, 1987; Imlay and Linn, 1986). In H_2O_2-resistant Chinese hamster fibroblasts, however, there was an increase in catalase activity (Spitz *et al*, 1988). There was good correlation between the degree of H_2O_2-resistance and catalase activity. Cells grown in 50μM H_2O_2 exhibited 4.5-fold catalase activity compared with wild-type cells and those grown in 400μM exhibited 32-fold resistance. This work suggests that in some cases mammalian cells are capable of increasing expression of catalase in response to the selective pressure mediated by its substrate, H_2O_2.

Metallothioneins are small sulphydryl-rich proteins that are expressed in all cells

of most species. Their physiological function is not completely understood although they do have a role in the metabolism and storage of zinc and copper. Expression of the metallothionein gene is inducible by, among others, cadmium, interleukin-1, glucocorticoid hormones, phorbol ester, UV radiation and Mitomycin C (Bauman *et al*, 1991). They have a molecular weight of between 6 and 7kDa and have a very high cysteine content. 30% of the amino acid residues are cysteine. In addition, the induction of metallothionein is probably a protective mechanism in mammals against cadmium chloride and other heavy metals. Metallothioneins bind heavy metals through clusters of thiolate bonds and they play a role in protection from oxidative stress. Metallothioneins have a strong scavenger activity against ·OH, the apparent biomolecular rate constant being of the order of $10^{12}M^{-1}s^{-1}$. Metallothioneins may also act as hydrogen atom donators to radical targets restoring them to their undamaged state (Thornalley and Vasak, 1985).

The glutathione-related enzymes are pivotal in maintaining the cellular redox state and these are discussed in detail in section 1.4. The export from cells of reactive species as free or conjugated forms also serves as a detoxification function. Enzymes such as glucose-6-phosphate dehydrogenase that are involved in the provision of reducing equivalents such as NADPH are also important in protection from oxidative damage. Proteins such as transferrin and caeruloplasmin that sequester transition metals, impede the Fenton reaction and so prevent the formation of ·OH. Thioredoxin can repair proteins that undergo disulphide linkage as a consequence of oxidative damage (Holmgren, 1985).

(b) Non-enzymatic antioxidants

Various non-enzymatic scavengers or "quenchers" are active in the cell. They are either lipophilic or hydrophilic. Tocopherol (vitamin E) is membrane-bound and accounts for most of the lipid-soluble antioxidant capacity in human erythrocytes (Burton *et al*, 1983). Tocopherol breaks the chain reaction of lipid peroxidation. Lipid peroxyl radicals that are formed during peroxidation react with other lipids at a rate constant of about $50M^{-1}s^{-1}$ but with tocopherol 10^4 or 10^5 times faster. Vitamin C is an important dietary antioxidant. It has been shown to be anticarcinogenic in rodents treated with UV radiation and benzo(a)pyrene (Hartman, 1982). Carotenoids are more hydrophilic than tocopherol and so provide protection in different cellular compartments (Krinsky, 1989). β-carotene is an efficient quencher of singlet oxygen. Glutathione is the major low molecular weight free radical scavenger and is discussed in section 1.3.

1.2.4 Clinical aspects of oxidative stress

The uncontrolled oxidation of tissues is a feature of many pathological processes. Reactive oxygen species interact with prostaglandins, leukotrienes, interleukins and other modulators of immune function. They have been implicated in the aetiology of many inflammatory conditions such as glomerulonephritis, vasculitis, autoimmune diseases and rheumatoid arthritis. The lung is particularly susceptible to oxygen toxicity which contributes to several pulmonary conditions including emphysema, adult respiratory distress syndrome and pneumoconiosis.

(a) Degenerative disorders and ageing

Oxidative stress has been implicated in the pathology of various degenerative disorders including some of the central nervous system and in ageing. The brain is particularly susceptible to oxidative damage because of its high rate of oxygen consumption and the non-regenerative nature of neurones. The brain also contains high levels of polyunsaturated fatty acids that act as substrates for lipid peroxidation. The effects of ageing on cellular function is thought to be a consequence of accumulating subcellular damage inflicted by activated oxygen species (Ames *et al*, 1981). However, evidence for the involvement of oxyradicals in ageing is, for the most part, indirect - mainly consisting of observations of the accumulation of peroxidation products in tissues and alterations in the components of the cellular antioxidant system. In studies using animal models and human autopsy material, brain levels of the end products of lipid peroxidation, malondialdehyde and lipofuscin, have generally been found to increase with age (Geremia *et al*, 1990). The aetiology of the degenerative condition, atherosclerosis, is multifactorial but it is believed that the underlying cause of this condition is endothelial damage by reactive oxygen species (Hennig and Chow, 1988).

(b) Anticancer treatments

The acquisition of resistance to cytotoxic drugs and ionising radiation by malignant cells is a major obstacle to improving cancer cure rates. Several commonly used chemotherapy drugs exert their cytotoxic effect partly by producing reactive oxygen species. Mitoxantrone and mitomycin C are examples of this. Sixty per cent of cancer patients receive radiation therapy and this is a form of treatment where cytotoxicity is also mediated by reactive oxygen species. It is true then that the means by which cells protect themselves against the damaging effects of oxygen may

contribute to the emergence of resistance to anticancer drugs and radiation. There are a number of commonly used quinone-containing antitumour agents. Adriamycin is an anthracycline antitumour antibiotic and is one of the most commonly used drugs in the treatment of cancer. Adriamycin undergoes bioreductive activation leading to the formation of drug and oxygen free radicals (Doroshow and Davies, 1986). Initial metabolism of adriamycin produces a hydroquinone which upon elimination of the C-7 glycoside forms a quinone methide, an electrophile that conjugates thiols, and thus leads to oxidative stress (Ramakrishnan and Fisher, 1986). Direct evidence that adriamycin produces ·OH and that this is partly responsible for cell killing was provided by Sinah *et al* (1987a, 1987b). A spin-trapping ESR technique demonstrated ·OH formation during the metabolism of adriamycin. It was also shown that adriamycin-induced cell killing was inhibited by SOD and catalase. Further evidence that adriamycin exerts its cytotoxic effect through oxyradicals was provided by Bachur *et al* (1978). Again, electron spin resonance spectroscopy was used to detect semiquinone free radical intermediates resulting from adriamycin metabolism in intact leukaemia L1210 cells and in isolated L1210 nuclei.

The most important clinical adverse effect of adriamycin is cardiotoxicity and it is this that limits the amount of the drug that can be administered. Cardiac toxicity is caused by quininoids and oxygen free radicals leading to disruption of the mitochondrial and sarcoplasmic reticulum of cardiac myocytes. Doroshow and Davies (1986) examined the effects of adriamycin and daunorubicin on $O_2^{-}\cdot$, H_2O_2 and ·OH production in preparations of beef cardiac submitochondrial particles. The anthracycline initiated oxygen radical cascade results in the peroxidation of mitochondrial lipid membranes.

(c) Hereditary disease

Genetically inherited diseases such as ataxia telangiectasia, Fanconi's anaemia and Bloom's Syndrome are characterised by an increase in cancer incidence and spontaneous chromosome breakage. There are indications in all three diseases of abnormalities in oxygen-metabolism. Cultured skin fibroblasts from patients with these diseases are more sensitive to agents that cause oxidative stress. Ataxia telangiectasia fibroblasts, for example, are more sensitive to ionising radiation and bleomycin. The cells of individuals with Fanconi's anaemia are unusually sensitive to mitomycin C and those from patients with Bloom's syndrome are sensitive to UV light (Cerutti, 1985).

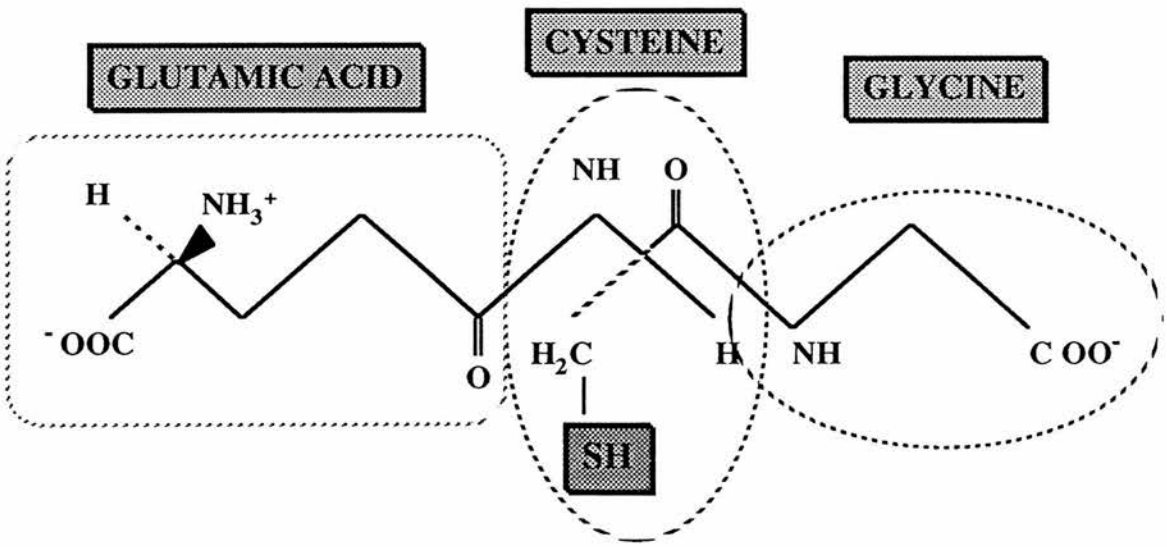
(d) Carcinogenesis

Oxygen radicals are thought to play a role in tumour development (Sun, 1990). They appear to do so mostly in the promotion phase of carcinogenesis during which genes that regulate cell differentiation and growth are affected. In the next step, progression, benign neoplasms can be stimulated to more rapid growth and ultimately malignancy. Evidence that reactive oxygen species are involved in carcinogenesis was provided by Borek and Troll (1983) who showed that the addition of SOD (but not catalase) to growth medium inhibited transformation of hamster embryo cells by X-irradiation and bleomycin. In addition, several non-enzymatic antioxidants protect from carcinogenesis. Vitamins C and E inhibit primarily the late steps of carcinogenesis and butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) inhibit the transformation of mouse skin cells promoted by PMA and benzoyl peroxide (Zimmerman and Cerutti, 1984).

1.3 THE ROLE OF GLUTATHIONE IN ANTIOXIDANT DEFENCE

Glutathione is ubiquitous and the predominant intracellular, non-protein sulphydryl in both prokaryotes and eukaryotes. It is a tripeptide consisting of one residue each of glutamate, cysteine and glycine. Its structure is shown in figure 1.3.

Figure 1.3 *Structure of glutathione*



The biosynthesis of reduced glutathione occurs in two successive ATP-requiring steps. First, γ-glutamylcysteine synthetase (γ-GCS) catalyses the formation of an amide linkage between cysteine and the gamma carboxyl of glutamate (equation 5):

$$\text{L-Glutamate} + \text{cysteine} + \text{ATP} \rightleftharpoons \gamma\text{-glutamyl-cysteine} + \text{ADP} + \text{Pi} \qquad (5)$$

Glutathione synthetase (GS) then catalyses the ATP-dependent conversion of γ-glutamylcysteine to form glutathione (equation 6):

$$\text{L-}\gamma\text{-Glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \rightleftharpoons \text{glutathione} + \text{ADP} + \text{Pi} \qquad (6)$$

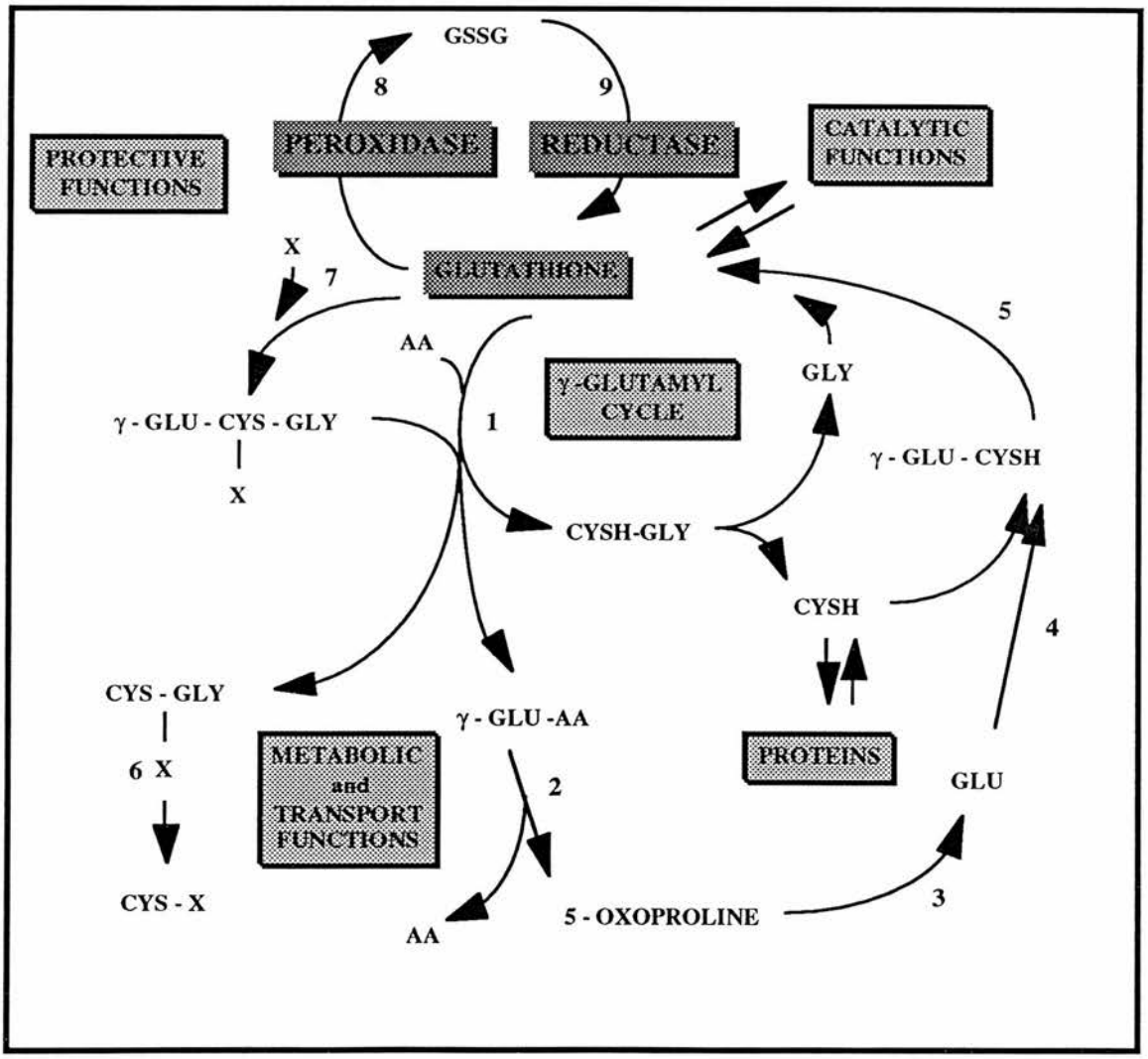
The rate limiting step in this process is the intracellular pool of cysteine and also, in the presence of excess cysteine, the activity of γ-GCS. The breakdown of GSH is catalysed by γ-glutamyl transpeptidase (γ-GT) which catalyses transfer of the γ-glutamyl moiety to acceptors such as cystine, glutamine, dipeptides or water. Glutathione acts as a cofactor for many glycolytic enzymes involved in amino acid catabolism and conversion. A role for glutathione in cell division has been postulated. Changes in GSH levels during the cell cycle have been described in various species. Why GSH levels change during the cell cycle is still at present unclear, but this may involve changes in the different forms of GSH. Exchange of GSH between the cytoplasmic and the smaller mitochondrial pool of GSH may also influence the levels of GSH.

The cellular processes in which glutathione takes part are summarised in Figure 1.4. In conjunction with the GSTs, glutathione conjugates with xenobiotics and compounds formed in metabolism. It acts as a source of reducing equivalents in synthesis and degradation of proteins, the formation of DNA precursors and the regulation of enzymes. In mammalian cells glutathione exists in at least three dynamically interchanging forms. Under normal steady state conditions, the majority of glutathione exists in the reduced form (GSH) with concentrations ranging from 0.5 (tissue culture cells) to 10mM (foetal liver) and turnover rates varying from 30 minutes (kidney) to 96 hours (mammalian erythrocyte). Oxidation of reduced glutathione, either non-enzymatically or through the action of glutathione peroxidase (GSHPx), yields GSSG (disulphide glutathione). Levels of GSSG are maintained at low concentrations within the cell, by NADPH-dependent reduction of GSSG by glutathione reductase (GR) or by efflux from the cell. A third form of GSH exists as mixed disulphides with both protein and non-protein sulphydryl compounds. A potentially significant fourth form of GSH can be found as thiol esters. The evidence for the occurrence and metabolism of these thiol esters is still preliminary. The

Figure 1.4 *Metabolism and functions of glutathione in the cell*

AA = amino acid; x = compounds that react with glutathione to yield conjugates such as those that lead to the formation of mercapturic acid, leukotrienes and compounds that are involved in other phases of metabolism. The enzymes involved in glutathione metabolism are listed below:

1. γ-glutamyltranspeptidase
2. γ-glutamylcyclotransferase
3. 5-oxoprolinase
4. γ-GCS
5. glutathione synthetase
6. dipeptidase
7. GST
8. glutathione peroxidase
9. glutathione reductase



Based on Meister and Anderson (1983) Ann. Rev. Biochem. **52,** 711-760.

identification, however, of at least three distinct thiol esterases in human liver perhaps suggest that they have some functional significance.

Reduced glutathione is characterised by its reactive thiol group and its γ-glutamyl bond where the glutamic acid is gamma linked to the cysteine residue (see Figure 1.3). It has been suggested that this unusual orientation reduces the susceptibility of the glutamyl-cysteine bond to proteolysis. This is exemplified by the finding that reactions of reduced glutathione (GSH) in mammalian cells can be divided into those involving the γ-glutamyl portion of the tripeptide or those of the sulphydryl moiety. Those involving the sulphydryl moiety can be further categorised into either oxidation-reduction reactions or nucleophilic conjugation reactions, in which the reduced sulphydryl reacts with an electrophile to form a thio ether.

In experimental models, GSH can be regulated by a variety of means. The administration of D-L-buthionine-S-R-sulphoximine (BSO), a potent specific inhibitor of γ-GCS can lead to a reduction in GSH levels. Conversely, cellular GSH levels can be increased by the administration of L-2-oxothiazolidine-4-carboxylate, γ-glutamyl cysteine or glutathione monoethyl esters (Meister *et al*, 1986). Such modulation can protect against cytotoxic drug-induced damage and also radiation damage. For example, treatment with BSO increases the sensitivity of mouse mammary tumour cells to peroxides and of human lymphoid cells to radiation (Dethmers and Meister, 1981). Regulation of GSH concentration is therefore important as this may influence the overall response of cells to oxidants. Glutathione is dispensable for the resistance of *E. coli* to both H_2O_2 and X-rays and even sensitises bacteria to these agents (Greenberg, 1986). Depletion of glutathione sensitises *E. coli* to redox-cycling agents.

1.4 THE ROLE OF GLUTATHIONE-RELATED ENZYMES IN ANTIOXIDANT DEFENCE

Glutathione-dependent enzymes are amongst those that protect cells against free radical damage and are implicated in cellular resistance to cytotoxic drugs. It is not known whether they play a similar role in radiation resistance.

1.4.1 Glutathione S-transferases

The glutathione S-transferases are a large and varied group of phase II xenobiotic-metabolising dimeric enzymes. GSTs have been detected in almost all living organisms including bacteria, yeast, plants, birds and mammals. They have the

capacity to catalyse the conjugation of electrophilic toxins with glutathione to more polar, readily excreted metabolites. They also sequester toxins through high affinity binding and remove toxic peroxides through organic peroxidase activity. Many isoenzymes have been described but the majority can be classified as belonging to the cytosolic multigene families alpha, mu, pi and theta or to the membrane-bound microsomal GST family (Mannervik *et al*, 1985; Morgenstern and De Pierre, 1983). GST-Pi is an isozyme that was first described in human placenta as an anionic protein and has been found and characterised for most human cell lines. Subsequently similar molecular forms of this enzyme class having similar molecular weights and immunoreactivity but distinct PI values were found in different species (human Pi, mouse MII and rat P).

There is evidence that the GSTs play a role in protection from oxidative stress. The overexpression of GST-Pi in a multidrug-resistant cell line pointed to the potential role of this isozyme in the detoxification of the free radical-induced damage caused by the quinone metabolites of adriamycin (Batist, 1986). Overall GST activity is increased in several cell lines selected for resistance to different antineoplastic agents that exert their toxicity at least partly through the generation of free radicals (Hayes and Wolf, 1988). Since GSTs, especially the alpha and pi isozymes, have peroxidase activity they may be involved in cellular protection against oxidative stress generated by, for example, ionising radiation (Ketterer and Meyer, 1989). Whelan *et al* (1989) found an increase in GST-Pi after fractionated irradiation of MCF-7 cells. These cells also showed 5-fold resistance to vincristine and 3-fold resistance to VP-16. GST-Pi induction by radiation is not a universal phenomenon, as a SuSa cell line (a human testicular teratoma) treated in the same way showed no significant alterations in GST isozyme profiles. In neither case was there any change in glutathione or glutathione peroxidase activity. Another way in which the GSTs may be involved in protection from oxidative stress is in the detoxification of quinones (Morgenstern *et al*, 1981).

The mechanism by which GST-Pi responds to changes in redox state are now partly understood. The protein products of the *c-fos* and *c-jun* genes form a heterodimeric complex that interacts with a DNA regulatory element known as AP-1 (activator protein-1). DNA-binding of the Fos-Jun heterodimer has been shown to be modulated by the redox status of the cell (Abate *et al*, 1990). Recent work involving the transfection of promoter deletion constructs into both drug-resistant and -sensitive MCF-7 cell lines demonstrated significantly increased transcriptional activity of GST-Pi promoter in resistant compared with wild-type cells (Moffat *et al*, 1994). DNA analysis revealed an AP-1 response element, TGACTCA, (at nucleotides 69-

63). These studies provide evidence that binding Jun-Fos to the AP-1 site of GST-Pi promoter is essential for transcriptional activation of GST-Pi.

1.4.2 γ-Glutamylcysteine synthetase

γ-GCS catalyses the first and rate limiting step of glutathione biosynthesis. The reaction is feedback inhibited by glutathione. The genes encoding rat and human γ-GCS have been cloned and sequenced (Yan and Meister, 1990; Gipp *et al*, 1992) and the protein purified from various sources (Seelig and Meister, 1984). The enzyme was first isolated from rat kidney and it consists of two subunits. One, of MW 72,614, which contains the binding sites for the substrates and for glutathione. The other subunit, MW 30,548, is catalytically inactive by itself but is essential for the function of the enzyme under physiological conditions. Feedback inhibition by GSH involves reduction of the enzyme as well as competition between glutathione and glutamate (Huang *et al*, 1993). The treatment of rats with sodium selenite increases the activity of liver γ-GCS - due to increased enzyme synthesis (Chung and Maines, 1981). Prolonged treatment of rats with methyl mercury hydroxide was associated with an increase in γ-GCS mRNA in kidney and this was thought to be the explanation for the 2-fold elevation in GSH that was also noted (Woods *et al*, 1992).

1.4.3 γ-Glutamyltranspeptidase

γ-Glutamyltranspeptidase (γ-GT) is one of the six enzymes of the γ-glutamyl cycle. The function of this cycle is to transport amino acids across the cell membrane. γ-GT is a membrane-bound enzyme and its active site is directed towards the outside of the cell. It is heterodimeric with a large (46-60kDa) and small unit (22kDa). γ-GT is responsible for initiation of degradation of GSH by the cleavage of the unique γ-glutamylcysteine peptide bond. Cysteinylglycine, a product of that reaction, is metabolised by a dipeptidase - and the free amino acids are then transported to inside the cell. γ-GT is elevated in some cell lines selected for resistance to cytotoxic agents. This is often associated with a concomitant elevation of GSH (Lewis, 1988a). This may be because γ-GT catalyses hydrolysis of GSH from the medium and thus increases the intracellular concentration of amino acids required for the synthesis of GSH.

1.4.4 Glutathione peroxidase

Glutathione peroxidase (GSHPx) catalyses the reduction of hydroperoxides

(ROOH) by GSH (equation 9):

$$ROOH + 2GSH \rightleftharpoons ROH + H_2O + GSSG \qquad (9)$$

The products generated are an alcohol, water and GSSG. Two types of GSHPx have been identified. A selenoenzyme containing selenium in the form of covalently bound selenocysteine at the active site and a non-selenium containing enzyme with negligible activity towards H_2O_2. This activity has been associated with the glutathione S-transferase family of enzymes particularly the alpha class but also the pi class. The selenium-dependent GSHPx was discovered in erythrocytes and has since been purified from a variety of sources including rat liver and lung, bovine erythrocytes and human placenta and erythrocytes. The bovine enzyme is a tetramer with a molecular weight of approximately 80,000. Selenium-dependent GSHPx has been also purified from human plasma. This enzyme, like the erythrocyte form, is tetrameric and contains four atoms of selenium per mole, but, unlike the erythrocyte protein, is glycosylated and subsequently has a molecular weight of 100,000. The entire mouse selenium-dependent GSHPx gene has been cloned and sequenced (Chambers *et al*, 1986). This work has shown that the selenocysteine at the active site of the enzyme is encoded by the stop codon TGA. The sequence of a cDNA clone encoding the human enzyme confirmed this finding.

The subcellular localisation of selenium-dependent GSHPx is important in its function, as catalase can also metabolise H_2O_2 as substrate. The relationship between catalase and selenium-dependent GSHPx has been studied. In hepatocytes catalase is primarily localised in the peroxisomes while selenium-dependent GSHPx is found both in the cytosol and mitochondrial matrix. The two enzymes therefore appear to have complementary localisations and functions in the cell. GSHPx activity has been demonstrated in all mammalian tissues. The selenium-dependent GSHPx is responsible for most of the activity, but in certain tissues, such as guinea pig liver, the selenium-dependent enzyme is absent. The ratio between the selenium-dependent and selenium-independent activity may vary, not just between animal species, but also from tissue to tissue within the same species. The factors involved in the tissue specific expression of selenium-dependent and selenium-independent GSHPx is unclear. It is known, however, that selenium concentrations can influence the relative expression of these enzymes. Apart from the effect of selenium, no other forms of regulation of GSHPx activity have been described.

The GSHPx enzyme functions to protect against toxic oxygen products generated by radiation, UV light and uncoupled mitochondria. In particular both

forms of GSHPx catalyse the reduction of lipid hydroperoxides and of peroxidised DNA. Mitochondrial selenium-dependent GSHPx is also involved in the regulation of mitochondrial substrate oxidations and protection of mitochondrial enzymes and DNA against lipid peroxidative damage.

There is a mutual protection of the major antioxidant enzymes by each other. GSHPx prevents peroxide-mediated inactivation of Cu/Zn-SOD (Hodgson and Fridovich, 1975) and SOD protects GSHPx from inactivation by superoxide (Blum and Fridovich, 1985).

1.4.5 Glutathione reductase

Glutathione reductase (GR) is a flavoprotein which catalyses the NADPH-dependent reduction of glutathione disulphide to glutathione (equation 10):

$$NADPH + H^+ + GSSG \rightleftharpoons NADP^+ + 2GSH \qquad (10)$$

GR has been purified from various sources including rat liver, yeast, porcine and human erythrocytes (Tutic *et al*, 1990). The GR gene for *E. coli* has been cloned and sequenced and is highly homologous to the human sequence (Greer and Perham, 1986). GR is found in almost all tissues and in all cells and functions to maintain glutathione levels in the reduced state. The enzyme exists as a homodimer with a molecular weight of 100,000 and contains one FAD molecule per subunit. Glutathione is not an essential metabolite in bacteria. Mutants (*gor*) that lack glutathione reductase have been isolated from *E. coli* and are found to grow normally (Davis *et al*, 1982). The enzyme has been shown to be induced by compounds such as TPA and metals such as selenium.

1.5 DEFENCE AGAINST OXIDATIVE STRESS IN PROKARYOTES

The powerful genetic techniques available for manipulations in bacteria have allowed progress in understanding the genetic and physiological responses of oxidative stress in bacteria. Many aerobic bacteria have multigene responses to oxidative stress and these are summarised below.

1.5.1 AppppN and oxidative stress

Small adenylylated nucleotides, which were at first thought to alert the cell to the

onset of metabolic perturbation, have been studied in *Salmonella typhimurium* (Bochner *et al*, 1984). These small molecules are able to influence gene expression and enzyme activity and in so doing redirect cellular metabolism to optimise the probability of survival. One alarmone, AppppA, is synthesised in cells following specific metabolic stresses. The highest concentration follows treatment with certain oxidants. It is not possible to detect AppppA in cells subjected to a wide range of other metabolic perturbations including deficiency of DNA, RNA, protein, adenosine or guanosine nucleotides, fatty acids, carbon or phosphate (Bochner and Ames, 1982). However, AppppA and similar molecules are induced by chemical oxidants such as 1-chloro-2,4-dinitrobenzene, H_2O_2, t-butyl hydroperoxide, cadmium chloride and a variety of quinones. Adenylylated nucleotides are synthesised as a side reaction of t-RNA synthetases and oxidative stress seems to be linked to adenylylated nucleotide synthesis via these enzymes (Zamecnik *et al*, 1966). For example, oxidants have been shown to inactivate leucyl-tRNA synthetase by reacting with a pair of sulphydryl groups located near the active site of the enzyme (Wiebauer *et al*, 1979). Synthetase could be reactivated *in vitro* by the addition of thiols. AppppA has been identified in mammalian as well as bacterial cells. Its function as redox-sensitive effector of cell growth appears to be highly conserved across species. Since AppppA induction lags behind that of the heat shock proteins it probably is not the signal for the initiation of the oxidative stress response but may modulate such a response.

1.5.2 Hydrogen peroxide response

When bacteria are exposed to H_2O_2 thirty to forty different proteins are induced. The induction of these proteins is associated with increased resistance to subsequent exposures to H_2O_2. This adaptive response to peroxide was first reported in 1977 (Hassan and Fridovich, 1977). The H_2O_2 response is also induced by UVA radiation and by superoxide-generating drugs both of which produce H_2O_2 as a secondary product. Of the forty proteins that are induced by H_2O_2, only a small number have been identified. The few that have been characterised have proven to be antioxidant enzymes.

A subgroup of nine proteins are co-regulated and are known as the OxyR regulon. The *OxyR* locus is at 89.5 min on the standard *E. coli* linkage map. Strains deleted for *OxyR* do not induce the nine proteins in response to treatment with H_2O_2 and are hypersensitive to H_2O_2 (Christman *et al*, 1985). Strains that show constitutively high resistance to peroxide (designated $OxyR^c$) show cross-resistance

to organic peroxides and to heat-induced killing. The proteins that are under the control of OxyR include catalase (encoded by *KatG*) and two alkyl hydroperoxide reductases (encoded by *ahpC* and *ahpF*). The latter are able to reduce hydroperoxides such as thymine hydroperoxide and linoleic hydroperoxide to their corresponding alcohols (Jacobson *et al*, 1989). The regulation by OxyR operates primarily at the transcriptional level. Storz *et al* (1990) showed that OxyR protein is directly activated by oxidation and that OxyR is rapidly and reversibly convertible between oxidised and reduced forms. It is believed that oxidative stress leads to direct conformational change in the OxyR protein that is then able to bind DNA. The nature of the protein change is not understood. An intraprotein disulphide does not appear to be the critical oxidation product because although OxyR does contain six cysteine residues, only one is essential for normal function of the protein (Christman *et al*, 1989). Another possibility is that a redox-active metal within the protein is responsible for sensing changes in cellular redox state. However, this is unproven.

OxyR seems then to act both as a sensor and transducer of oxidative stress. It is able to bind to three different DNA sequences that are all located upstream of *OxyR*-regulated promoters (Tartaglia *et al*, 1989). It binds its DNA targets by a helix-turn-helix recognition motif and triggers transcription either by contacting RNA polymerase bound to promoters or facilitating promoter-binding by the enzyme. OxyR exerts a negative autoregulatory effect on the promoter of its own gene (Tao *et al*, 1991). This could serve as a mechanism to down-regulate the response.

Salmonella typhimurium strains with deletions of *oxyR* showed 10- to 55-fold higher frequencies of spontaneous mutagenesis (Storz *et al*, 1987). The largest increase in mutation frequency was observed for T.A to A.T transversions (40- to 146-fold) which is the base-substitution most often seen with chemical oxidants (Levin *et al*, 1992). That the increased mutation frequency was due to the lack of expression of the OxyR-controlled genes was demonstrated by the addition to the deletion strains of a plasmid carrying the OxyR-regulated genes, *katG* and *ahp*. This manoeuvre reduced the mutation rate. Furthermore strains carrying an *oxyR* mutation that results in overexpression of the protein show approximately half the number of spontaneous mutations as wild type strains. Thus the bacterial stress response to H_2O_2 prevents the mutations caused by peroxides that are the inevitable result of normal aerobic growth.

1.5.3 Superoxide response

Treatment of bacteria with superoxide-generating compounds such as paraquat

and menadione leads to the induction of forty proteins. In addition, because $O_2^{-\cdot}$ dismutates to give H_2O_2, the 40 peroxide-inducible proteins are also induced. In *E. coli* nine of the forty superoxide-inducible proteins constitute a coregulated group under the control of a two-gene locus named *soxRS* (Amábile-Cuevas and Demple, 1991; Greenberg *et al*, 1990). The *soxRS* locus has been localised to 92.2 min on the genetic map for *E. coli*. The *soxRS* regulon was discovered by the selection of mutants with constitutively elevated resistance to menadione (designated *soxRS*^{c}) (Greenberg and Demple, 1989). These mutants showed cross-resistance to organic peroxides and bleomycin and increased levels of several enzymes known to be inducible by superoxide-generating agents, including Mn-SOD *(sodA)*, the DNA repair enzyme endonuclease IV (*nfo*), glucose-6-phosphate dehydrogenase (zwf) and six other proteins of unknown function including those controlled by *soi17/19* (superoxide-inducible) and *soi28* loci. The addition of paraquat to *E. coli* leads to a 10- to 20-fold increase in endonuclease IV within one hour (Levin *et al*, 1988). Mutation of *nfo* increases sensitivity of *E. coli* to many DNA damaging agents. In most cases this effect is only readily apparent in strains in which exonuclease III is also mutated (Cunningham *et al*, 1986). Glucose-6-phosphate dehydrogenase replenishes NADPH for use by glutathione reductase or following redox-cycling. *Soi17/19* and *soi28* have been shown to contribute to paraquat-resistance in *E. coli*. As well as the forty inducible proteins there are three proteins that are repressed by superoxide-generators in wild type cells and these are also repressed in *soxR*^{c} strains (Greenberg *et al,* 1990).

As well as being resistant to oxidative stress, *soxR*^{c} strains show increased resistance to several antibiotics (namely chloramphenicol, quinolones, tetracycline and ampicillin) (Greenberg *et al*, 1990). This unusual feature of the superoxide stress response was found to be due to the decreased synthesis of OmpF, an outer membrane porin. The physiological role of OmpF is to facilitate the uptake of various solvents by providing a hydrophilic transmembrane channel. Diminished expression of OmpF results in exclusion of compounds such as antibiotics. It is unknown whether it has a specific role in antioxidant defence. Another soxR-regulated change is an unusual post-translational modification of the ribosomal protein S6. Several glutamate residues are added to the C-terminus. The enzyme that catalyses this modification has not been identified but it is controlled by soxR and the changes it causes have been shown to render ribosomes more resistant to attack by oxidants.

Gene regulation in the SoxRS system occurs primarily at the transcriptional level. Deletion analysis has been used to demonstrate that both *soxR* and *soxS* are

involved in controlling the soxRS regulon. The predicted soxR and soxS proteins are homologous to other transcription factors. SoxR is homologous to MerR transcription factors which are regulators of resistance to mercury (O'Halloran *et al*, 1989). Like MerR, SoxR contains four cysteine residues clustered near the C-terminus of the protein. MerR is triggered when Hg^{2+} is bound by the thiol groups of the four cysteines. It is possible that the cysteine residues of SoxR act as metal-binding ligands and that this is the mechanism by which the protein is activated. SoxS is homologous to the AraC family of transcription factors.

It has been postulated that SoxR and SoxS act together in a mini-cascade to regulate the *soxRS* regulon. It is proposed that a cellular signal (possibly superoxide or other redox effects such as NADPH-depletion) activates pre-existing SoxR protein by causing a conformational change. This then triggers the expression of the *soxS* gene, and so the amount of SoxS protein increases. This then binds to the promoters of the various SoxRS-controlled genes (Demple and Amábile-Cuevas, 1991). The promoters controlled by SoxRS are still only partly characterised but there are known to be common elements in the promoters of the *sodA*, *nfo* and *zwf* genes.

1.5.4 Stationary phase/starvation response

The induction of a group of 20 to 30 proteins on cessation of cell growth contributes to protection from oxidative stress. There are subtle differences in the patterns of protein induction according to which nutrients are withheld. Examples of antioxidant enzymes that are induced by starvation are catalase and exonuclease III (Sak *et al*, 1989; Loewen and Triggs, 1984). Exonuclease III is the product of the *xthA* gene and is the major apurinic/apyrimidinic exonuclease activity found in *E. coli*. It has been shown to remove replication blocks from the 3' termini of oxidised DNA. Null mutation of the *katF* locus prevents the synthesis of proteins associated with the starvation response. The predicted KatF protein shows homology to the σ factors that function as subunits of bacterial RNA polymerase to guide the enzyme to specific promoters. The induction of *katF* occurs in response to spent medium from stationary phase cells or to aromatic acids such as benzoate.

1.6 DEFENCE AGAINST OXIDATIVE STRESS IN EUKARYOTES

The response of mammalian cells to oxidative stress has been less well characterised than that of prokaryotes. Nearly all aerobic cells be they prokaryotic or

eukaryotic possess SODs, catalases and peroxidases which destroy active oxygen species. In addition, some eukaryotic and bacterial DNA repair enzymes specific for oxidative damage share extensive homology. There are however fundamental differences in the control and regulation of the antioxidant processes in prokaryotic and eukaryotic cells. Oxyradicals do not, in general, induce transitory expression of antioxidant defence enzymes in eukaryotes. For example, the cytokines such as tumour necrosis factor and interleukin-1 are potent inducers of Mn-SOD in mammalian cells whereas oxidative stress has only a modest effect (Wong and Goeddel, 1988). This may be because most mammals live in an environment in which atmospheric gases are more or less constant whereas bacteria may be subjected to sudden changes in oxygen concentration. An exception is the sequestration and activation of neutrophils in mammals in response to injury. These produce large fluxes of reactive oxygen species. It would make sense for the signals (usually cytokines) that induce inflammatory responses to also induce those enzymes that are capable of detoxifying oxyradicals. In this way cells close to the site of inflammation would be protected from oxidative stress.

Whether eukaryotic oxidation stress responses include proteins with functions analogous to those seen in *E. coli* awaits further work. However, a number of genes that are specifically regulated by oxidative stress have been identified in the last few years.

1.6.1 Heme oxygenase

In the 1980s a 32kDa stress protein which was inducible by a number of stresses but particularly H_2O_2 and UVA was observed. It was known to be inducible by heavy metals and thiol reactive agents in both normal and neoplastic cells (Caltabiano *et al*, 1986). Keyse and Tyrrell (1989) identified the inducible gene as encoding heme oxygenase. Heme oxygenase (HO) is a monomeric protein that exists as two isoenzymes. It plays an important physiological role in heme degradation but is also inducible in different tissues to different extents by a large number of agents which include heme compounds, heavy metal ions, organic solvents such as benzene, halogenated hydrocarbon drugs such as cyclophosphamide, X-irradiation, sulphydryl compounds such as sodium arsenite, diethyl maleate and oxidative stresses such as UVA radiation and hydrogen peroxide. Heme oxygenase is induced by heat in rats although not in humans and is thought to protect against oxidative stress in a number of ways. Firstly, by turning over respiratory mitochondrial cytochromes which may be inactivated by oxidative stress. Secondly, by the removal of potential

chromophores involved in radical formation by UVA radiation. Finally, the product of its action on haemoproteins is biliverdin and also, in the presence of biliverdin reductase, bilirubin. Unconjugated bilirubin is an efficient scavenger of singlet oxygen and is able to react with superoxide anion and peroxyl radicals (Stocker *et al*, 1987).

Induction of HO is due to an increase in the rate of transcription. An important signal for the regulation of HO is the availability of glutathione. UVA in small dose will induce HO when glutathione is depleted using buthionine sulphoximine. Diamide oxidises glutathione and leads to elevation of HO. UVA radiation, hydrogen peroxide and sodium arsenite all induce HO and are also capable of depleting cellular GSH.

The exposure of cells to iron chelators (desferrioxamine or o-phenanthroline) prior to treatment with H_2O_2 or UVA attenuates the induction of heme oxygenase (Keyse and Tyrrell, 1989). This is probably because iron chelators inhibit the Fenton reaction and therefore reduce the production of ·OH. The induction of heme oxygenase by sodium arsenite was completely unaffected by pre-treatment with iron chelators. UVA and H_2O_2 both act through the formation of free radicals whereas sodium arsenite reacts with cellular sulphydryl groups, a process which iron chelation would not be expected to effect.

Paradoxically, HO is inducible by hypoxia as well as by oxidative stress. Hypoxia induces a group of proteins which undergo enhanced rates of synthesis-depending on the severity and duration of the stress (Heacock and Sutherland, 1986). Five major oxygen-regulated proteins (ORPs) have been documented. Two, ORP80 and ORP100, are probably the same as glucose regulated stress proteins GRP78 and GRP94. ORP33 is thought to be HO. Murphy *et al* (1991) showed induction of HO protein in Chinese hamster ovary cells maximally at 12 hours of hypoxia. Hypoxic stress leads to depletion of glutathione and this may be the reason why heme oxygenase induction is triggered. Induction of ORPs (oxygen-regulated proteins) is maximal in hypoxic A341 and CaSki human squamous carcinoma cells after 12 hours of hypoxia when GSH is also at its lowest (Kwok and Sutherland, 1989).

1.6.2 Clone 100

In 1992 Emslie and Keyse reported the finding of a human gene which was induced by oxidative stress and heat shock and which encoded a protein-tyrosine phosphatase. The gene which has been designated, CL100, specifies a protein of molecular weight 39.3kDa. The carboxy terminal portion of the CL100 polypeptide

contains a single copy of the highly conserved active site sequence of protein-tyrosine phosphatases (PTPases) and the purified protein encoded by the CL100 open reading frame expressed in bacteria has intrinsic phosphatase activity. CL100 is strongly induced by menadione, UVA and hydrogen peroxide. Unlike heme oxygenase, CL100 is significantly induced by heat shock. CL100 is not induced by sodium arsenite which produces oxidative stress by interacting with glutathione. CL100 was not found to be significantly inducible by DNA-damaging treatments such as UVC, cisplatin and ionising radiation and so DNA damage does not appear to trigger induction of this gene.

Protein-tyrosine phosphorylation is important in cell-cycle control. Considering the similarity between CL100 and protein-tyrosine phosphatase, the induction of this gene may play a regulatory role in the mammalian cellular response to oxidative stress.

1.6.3 Transcription factors

(a) NF-κB

NF-κB is a eukaryotic inducible transcription factor which is involved in the regulation and transcription of a variety of inflammatory and immune response genes. DNA-binding of NF-κB has been shown to be stimulated by a wide variety of stimuli including phorbol esters, inflammatory cytokines, UV radiation, ionising radiation, viral and bacterial proteins, lipopolysaccharides and double-stranded RNA. NF-κB forms a complex with IκB in the cytoplasm of unstimulated cells. The NF-κB complex undergoes post-translational modification. IκB is phosphorylated through the action of protein kinase C (PKC) or other kinases (Ghosh and Baltimore, 1990) and this is thought to be mediated through reactive oxygen species. This phosphorylation dissociates NF-κB from IκB and allows NF-κB to migrate to the nucleus where it activates its target genes. However, not all activation of NF-κB is PKC-mediated since depletion of PKC by chronic PMA treatment does not affect NF-κB activation by TNF. NF-κB binds to a decameric sequence motif in the promoter and enhancer elements of genes. It can very rapidly activate genes because the protein is already present in unstimulated cells and requires for its activation the release of IκB.

(b) Jun/Fos

Oxidative stress causes rapid induction of early response genes such as *c-jun*, *c-*

fos and *c-myc* (Crawford *et al*, 1988). AP-1 is composed of the *jun* and *fos* gene products which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. Several oxidants including H_2O_2 and UV radiation can induce AP-1 activation (Devary *et al*, 1991). Oxidation of a single cysteine residue in the DNA-binding domain of Jun and Fos proteins is thought to control DNA-binding of the two proteins *in vitro* (Abate *et al*, 1990). Smeyne *et al* (1993) have shown c-Fos induction at sites of apoptotic death. The activity of AP-1 has been shown to be strongly promoted under reducing conditions following treatment with, for example, thioredoxin (Schenk *et al*, 1994) or the metal chelator and radical scavenger, pyrrolidine dithiocarbamate (PDTC) (Meyer *et al*, 1993).

1.6.4 Oncogenes

There is accumulating evidence that reactive oxygen species play a prominent role in precipitating programmed cell death and the proto-oncogene, *bcl-2*, is now thought to control this process through the regulation of antioxidant pathways (Hockenbery *et al*, 1993). *Bcl-2* is probably only one element in a cascade of genes regulating apoptosis. It appears to act close to the final irreversible step where the various afferent pathways converge and at which the effector processes are activated (Wyllie, 1994). Expression of Bcl-2 protein prevents induction of apoptosis by a variety of oxidative stresses (including ionising radiation, heat shock and inhibition of glutathione synthesis) (Korsmeyer, 1992). Mitochondria are believed to be a major site of reactive oxygen species production and subcellular fractionation studies indicate that Bcl-2 is as an integral mitochondrial membrane protein having a carboxy-terminal membrane anchor and is also localised to the nuclear and endoplasmic reticulum membranes. The proximity of Bcl-2 to lipid membranes facilitates its inhibition of lipid peroxidation (Hockenbery *et al,* 1990).

In interleukin-3 dependent FL5.12 cells menadione caused massive cyanide-resistant oxygen consumption (Hockenbery *et al*, 1993). This is unaltered by overexpression of Bcl-2. Oxygen consumption that exists after a cyanide block of cytochrome oxidase function represents oxygen consumption from pathways such as superoxide anion generation and does not normally exceed 1-2% of cellular oxygen consumption. Bcl-2 does protect cells from menadione induced cell killing. That is, it protects after the superoxide anion stage probably by protecting against the effects of H_2O_2 and $\cdot OH$. Bcl-2 shows no capacity to block free radical generation. Hydrogen peroxide generation is unchanged by the overexpression of Bcl-2 in cells that are induced to undergo apoptosis by the withdrawal of interleukin-3. In other

words, peroxides continue to be formed but Bcl-2 somehow blocks their damaging effects.

1.7 QUINONE CHEMISTRY

Much of the experimental work carried out to elucidate the cellular response to oxidative stress in both prokaryotes and eukaryotes has made use of quinone compounds. Many members of this class of chemicals are capable of generating a flux of $O_2^{-\cdot}$ and thus disturbing cellular redox state. Since much of the work described in this thesis involves the use of menadione a short summary of quinone chemistry follows.

1.7.1 Physiological and pharmacological role of quinones

Quinones are diketones that are derived from aromatic compounds and the two carbonyl groups may be in the same or different rings. Quinones are widely distributed in nature being found in higher plants, fungi and bacteria as well as throughout the animal kingdom (Thompson, 1971). They can exist in various redox states and can participate in electron transport processes such as mitochondrial respiration and photosynthesis. The first commercially useful quinones, chloranil and dichlore, were developed as fungicides in the 1940s. Their anti-fungal action is due to the binding of quinone to protein thiol and amine groups and to the disruption of mitochondrial electron transport. Some antimalarial drugs are naphthoquinones whose mode of action is to compete with ubiquinones which leads to interference with mitochondrial electron transport. Many plants contain quinones these being formed by the action of peroxidases on phenols. Their function in plants seems to be as bactericidal agents. Quinones are produced as a result of oxidation of environmental aromatic hydrocarbons that are present in, for example, cigarette smoke, car and diesel exhaust and are also found in many food stuffs (Ames, 1983). They are therefore of general toxicological importance. It is possible that they are carcinogenic. The solvent, benzene, for example, is converted in the liver to the phenol, hydroquinone, which then undergoes oxidation (by myeloperoxidases) and reaches the bone marrow where it has a leukaemogenic effect.

Quinones form the second largest class of cancer chemotherapy agents (after the alkylating agents). The quinones in clinical use are extensively substituted anthraquinones, *p*-benzoquinones or naphthoquinones (Powis, 1987). The anthraquinone glycoside antibiotic, adriamycin, was isolated in 1963 and found to

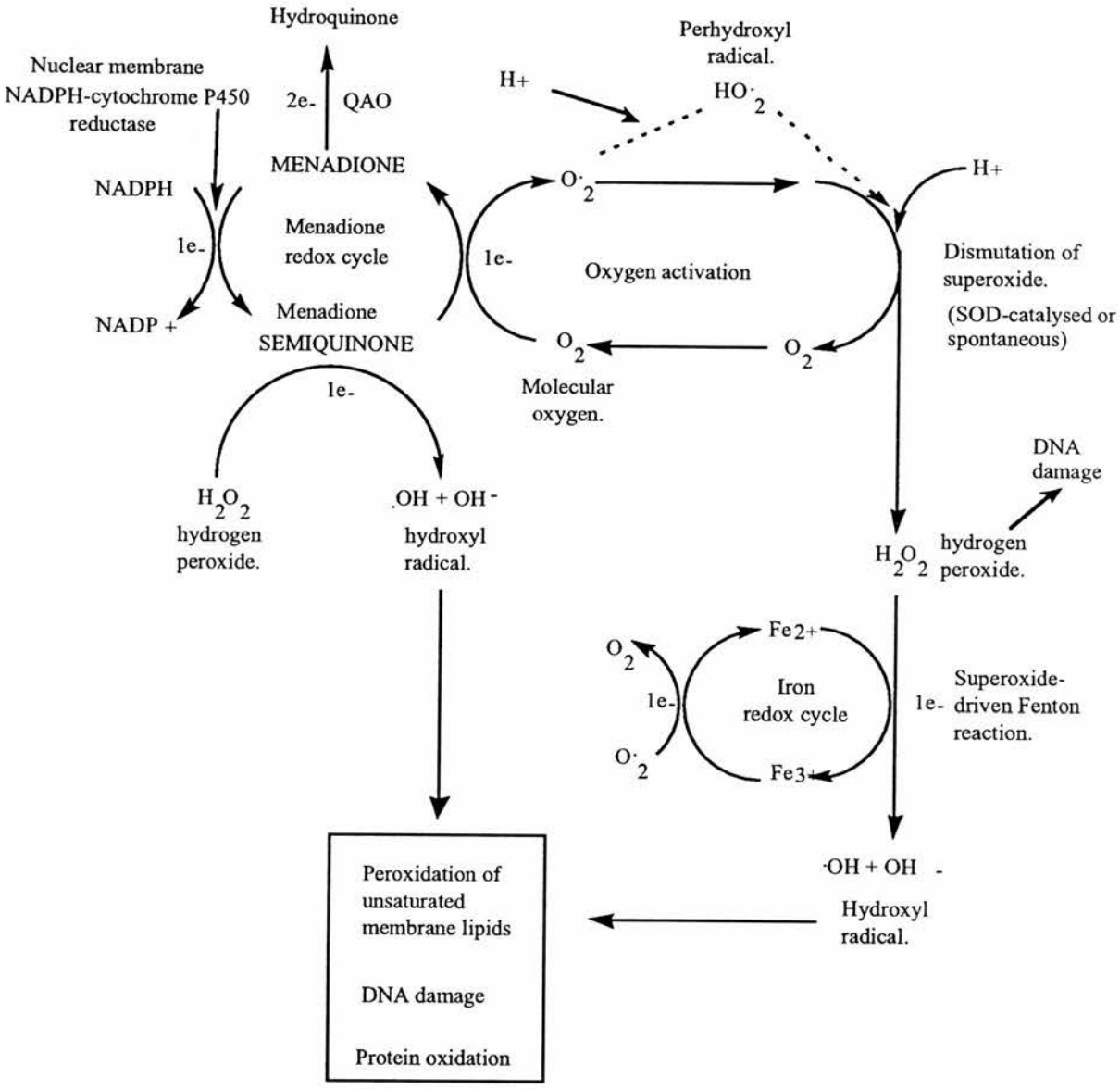
have antileukaemic activity. It is now one of the most commonly used agents in cancer therapy. The most important side-effect of adriamycin, cardiac toxicity, has been attributed to futile redox-cycling of the drug in the myocyte leading to oxygen activation and cytotoxicity.

1.7.2 Quinone cytotoxicity

The mechanisms of quinone cytotoxicity are summarised in Figure 1.5. The isolated hepatocyte has been used extensively as a model to study quinone toxicity. Badr *et al* (1989) showed that perfusion of the liver with the model quinone, menadione, leads to a concentration-dependent increase in oxygen uptake and periportal necrosis. The periportal area, being well supplied with blood, is a particularly oxygen-rich area of the liver and this may explain why it is particularly susceptible to quinone-induced damage. Toxicity was preventable by simultaneous administration of desferrioxamine (an iron chelator), cianidanol and allopurinol (a xanthine-oxidase inhibitor). Bellomo *et al* (1990) also used the isolated rat hepatocyte as a model target. Menadione was found to cause cyanide-resistant respiration, oxidation of glutathione and loss of cell viability.

One of the effects of the quinones is glutathione-depletion which results in macromolecules such as membrane-related and other proteins being alkylated by the quinone (Hoffman *et al*, 1985). Some toxic quinones are formed by oxidation *in vivo*. The neurotransmitters in the central nervous system, dopamine, adrenaline and noradrenaline, for example, undergo autooxidation, in the presence of transition metals, to form quinones as well as melanin, superoxide and hydrogen peroxide. Quinones can undergo stepwise one-electron reduction to their semiquinone free radical and then hydroquinone (Figure 1.6). During the autooxidation process, dioxygen is itself reduced to the superoxide radical, $O_2^{-\cdot}$. Thus the one-electron reduction of quinones to semiquinones and the subsequent autooxidation of the semiquinone to the quinone can yield large quantities of $O_2^{-\cdot}$. This non-stoichiometric, reduction-oxidation process is known as redox-cycling, and is largely responsible for the oxidative injury caused by quinones. Quinone redox-cycling is catalysed by a number of different flavoenzymes including NADPH-cytochrome P-450 reductase, NADPH-cytochrome b5 reductase and NADH-ubiquinone oxidoreductase. The relative ability of each of these enzymes to catalyse the one-electron reduction of a given quinone is related to the one-electron reduction potential of the quinone rather than to its structural features or lipid solubility. NADPH-cytochrome P-450 reductase and NADPH-cytochrome b5 reductase use

Figure 1.5 *Redox-cycling by menadione*



Adapted from Mimnaugh *et al* (1985) Cancer Res. **45;** 3296-3304

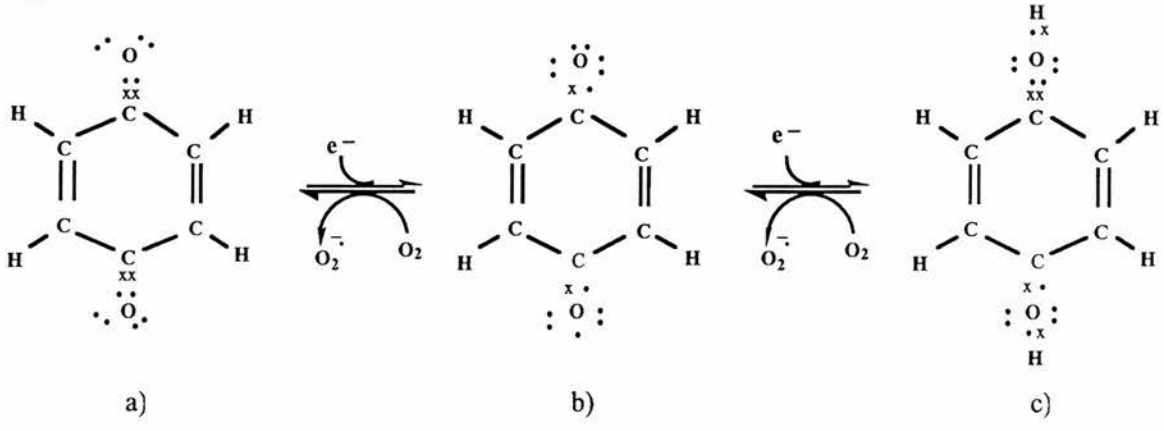
quinones as electron acceptors and catalyse a one electron reduction.

An alternative pathway of quinone metabolism exists and this consists of a two electron reduction, catalysed by quinone acceptor oxidoreductase (formerly termed DT-diaphorase). The product of this reaction is a hydroquinone, which is far less reactive than the parent quinone. The hydroquinone does not take part in redox-cycling and so does not induce oxidative stress. It is readily conjugated to UDP-glucuronic acid for export from the cell (Thor *et al*, 1982). This alternative pathway of quinone metabolism is therefore thought to be protective. Quinone acceptor oxidoreductase (QAO) is inducible by 3-methylcholanthrene and it is inhibited by dicoumarol. The induction of QAO by polycyclic hydrocarbons is governed by the aromatic hydrocarbon-responsive Ah locus (Nebert *et al*, 1990). The metabolism of quinones is associated with the consumption of intracellular glutathione and modification of protein thiols. Quinone-induced thiol-consumption is due to oxidation of glutathione to disulphide glutathione and so is linked to H_2O_2 metabolism by glutathione peroxidase. Disulphide glutathione is subsequently reduced back to GSH by glutathione reductase at the expense of NADPH. However, the capacity of cells to regenerate NADPH becomes exhausted, disulphide glutathione accumulates and a state of oxidative stress prevails.

Bellomo *et al* (1990) compared the metabolism of menadione which is a mixed alkylating/redox-cycling quinone with that of p-benzoquinone (an alkylating quinone) and 2,3-dimethoxy-1,4-naphthoquinone (a redox-cycling agent). Both menadione and 2,3-dimethoxy-1,4-naphthoquinone result in GSH depletion and synchronous elevation of GSSG whereas p-benzoquinone only depletes GSH. Pretreatment of hepatocytes with BCNU (which inhibits glutathione reductase) potentiated the depletion of GSH and elevation of GSSG. With the alkylating agent, p-benzoquinone, GSH loss is not associated with GSSG formation and BCNU pretreatment fails to accelerate depletion of glutathione or elevation of GSSG. Protein mixed disulphides are formed in a dose-dependent manner following menadione metabolism of 2,3-dimethoxy-1,4-naphthoquinone in the cytosolic fraction and lesser amounts in microsomes and mitochondria. Mixed disulphide formation increases with BCNU (because glutathione reductase may be involved in removing GSH-mixed disulphides) (Bellomo *et al*, 1987). Mixed disulphides are not formed during metabolism of p-benzoquinone in either control or BCNU-treated hepatocytes.

Figure 1.6 *One- and two-electron reduction of quinones*

Stepwise one-electron reduction of (a) quinone to its (b) semiquinone free radical and (c) hydroquinone. The autooxidation of the two reduced forms is also shown with dioxygen as the oxidising agent. During this autooxidation process the dioxygen itself is reduced to the superoxide anion.



a) b) c)

1.7.3 Mutagenicity of quinones

Cells are particularly sensitive to oxidative DNA damage. Hyperbaric oxygen, γ-irradiation, H_2O_2 and $O_2^{-\cdot}$ are all oxidants that have been shown to be mutagenic. They cause chromosome deletions, dicentric forms and sister chromatid exchange (Cerutti, 1985). An early study showing the mutagenic effect of naphthoquinones including menadione was carried out by Tikkanen *et al* (1983). Chesis *et al* (1984) investigated the mutagenicity of a number of quinones including menadione. QAO is selectively inhibited by dicoumarol and this allowed assessment of the contribution of this pathway to the mutagenic activity of different quinones. Menadione was approximately twice as mutagenic when dicoumarol was present. Thus inhibition of QAO did enhance the mutagenicity of menadione - confirming the protective role of this pathway. Also mutagenicity was greater in the presence of NADPH compared with NADH indicating the importance of NADPH-cytochrome P-450 reductase in mediating quinone mutagenicity. Mutagenesis was inhibited by the addition of SOD or catalase to the system indicating that mutagenicity was indeed due to the formation of oxyradicals.

1.8 SUMMARY AND AIMS OF THESIS

Organisms exhibit the ability to adapt to environmental challenge by the initiation of homeostatic responses. At the cellular level there are many mechanisms by which the cell can respond to changes in its normal growth environment. Examples of this include temporary or reversible change in enzyme structure or function by which certain metabolic pathways are modified. Alternatively, there may be more long-term changes that involve permanent alterations in the pattern of gene expression, thereby facilitating the adaptation of the cell to its new circumstances. It is apparent that most metabolic pathways are altered in some way by oxidative stress.

Reactive oxygen species may be generated endogenously through respiration and other metabolic processes or result from chemical and physical agents applied to the cell. Very complex and orchestrated cellular responses to reactive oxygen species have now been described in bacteria. The various components of these responses prevent, intercept and repair the damage caused by reactive oxygen species. Two major oxyradical-responsive bacterial transcription factor systems (OxyR and Sox RS) have been investigated in detail. However, many of the proteins induced either by peroxide or superoxide anions remain to be identified. An understanding of cellular defence against oxidative stress is clinically important because oxidative damage has been implicated in many pathological and degenerative conditions. Since many anticancer therapies owe their efficacy to the damaging effects of reactive oxygen species, the means by which cells protect themselves from oxidative damage are likely also to be the mechanisms that cause chemo- and, perhaps, radio-resistance.

The aims of the thesis were to:

1. Establish and characterise mammalian cell lines that are adapted to growth in conditions of oxidative stress,

2. Determine whether there is a functional overlap of the oxidative stress response with adaptive responses to other forms of stress in mammalian cells,

3. To elucidate the enzymatic and non-enzymatic defence mechanisms used by mammalian cells to withstand oxidative stress,

4. To study mechanisms of transcriptional regulation of oxidative stress-inducible genes in mammalian cells.

CHAPTER TWO

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest grade of purity available.

2.1 CELL CULTURE

2.1.1 Growing and routine maintenance of cell lines

Parental cell lines were taken from laboratory stocks that had been stored in liquid nitrogen. The cell lines used were CHO-K1, derived from Chinese hamster ovary and EJ-WT, derived from human transitional cell carcinoma of the bladder. To thaw cells, cryotubes were agitated gently in a water bath at 37°C immediately after removal from liquid nitrogen. Cells were carefully transferred by means of plastic pastettes from the cryotube to a universal tube containing 5ml of appropriate medium. Cells were washed twice in medium to remove DMSO and then transferred to a $25cm^3$ culture flask (Nunc). They were allowed to adhere overnight before refeeding with fresh medium.

CHO-K1 and its sublines were grown in alpha-Minimum Essential Medium (α-MEM) (Gibco). EJ-WT and its sublines were grown in Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco). Media were supplemented with 10% (v/v) foetal calf serum (Gibco), streptomycin (100μg/ml), penicillin (100iu/ml) (Gibco) and glutamine (2mM). Cells were cultured as a monolayer at 37°C at 100% humidity in an atmosphere of 5% CO_2. Cell lines were cultured for no more than ten passages. The exception to this was the isolation of menadione-resistant cell lines by continuous exposure to drug, a procedure which required many passages (see section 2.1.2.b). After ten passages, cells were discarded and a fresh aliquot of cryopreserved cells taken from frozen stocks. This prevented the emergence of phenotypically altered cells, which can occur after protracted culture.

During routine culture, spent medium was removed and replaced with fresh medium every two to three days. Cells were maintained in logarithmic growth phase by frequent subculture. To do this, medium was poured off and cells washed three times with PBS (140mM NaCl, 2.7mM KCl, 8mM sodium phosphate pH 7.4) (Oxoid). Cells were harvested using sterile 0.1% (w/v) trypsin (Difco Laboratories) and 0.001% (w/v) versene (EDTA) in a ratio of 1:1. Sufficient trypsin/versene was

added to cover the cells. Most cells detached from the flask within 5 minutes. The flask was shaken gently to dislodge any remaining adherent cells. The trypsin/versene/cell mixture was transferred to a universal flask and the cells washed three times with medium before being suspended in 10ml of fresh medium. The cell suspension obtained in this way was divided into equal volumes and seeded into new flasks. CHO cell lines were subcultured every two days and EJ-WT cell lines every three days.

For cryopreservation, cells were harvested using trypsin/versene. The cell suspension was centrifuged at 1800g and the supernatant of trypsin/versene removed. Approximately 100 x 10^6 cells/ml were resuspended in 2ml of freezing mix consisting of 90% (v/v) new-born calf serum (Gibco) and 10% (v/v) DMSO (Sigma). The freezing mix and cells were pipetted into pre-cooled cryotubes. Cells were transferred immediately to liquid nitrogen.

2.1.2 Strategies for isolation of menadione-resistant cell lines

(a) Pulsed treatments of menadione

Wild-type cells in logarithmic growth phase were treated with menadione at 40μM for 48 hours. Treated cells were allowed to recover in drug-free medium for between four and eight weeks. Once there were sufficient cells, several aliquots were frozen. Some were continued in culture and exposed again to 40μM menadione or to the next incremental concentration (60μM). This procedure was repeated using 80μM and then 100μM menadione. Cells were exposed to a total of six pulses of drug. The cell lines derived in this way were designated CHO-MRs40, CHO-MRs60, CHO-MRs80, CHO-MRs100, EJ-MRs40, EJ-MRs60, EJ-MRs80 and EJ-MRs100.

(b) Continuous exposure to drug

Menadione-resistant cells were isolated by continuous exposure of cells to menadione. A preliminary experiment was carried out to determine the LD_{75} concentration of menadione for both parental cell lines. This was found to be 20μM. Menadione was added at this concentration to logarithmically growing parental cells. When cells had developed tolerance to 20μM menadione the concentration was increased to 30μM and then to 40μM. Menadione was made up to a concentration of 10^{-2}M in ethanol. This stock drug solution was made up weekly and stored at 4°C. Cells were allowed to adapt for several passages at each concentration before the next incremental dose was used. The cell lines obtained in this way were designated CHO-MRc20, CHO-MRc30, CHO-MRc40, EJ-MRc20, EJ-MRc30 and EJ-MRc40.

Thirty eight passages were required to isolate CHO cells which grew in 40μM menadione. Several attempts were made to grow cells in 50μM menadione but this was not possible. The resistant cell lines were maintained by continuous exposure to menadione. Cells were grown in drug-free medium for 3 to 4 days before use in experiments.

(c) Pretreatment with mutagen

A third strategy for obtaining menadione-resistant cells was employed. Cells were mutagenised using ethyl methane sulphonate (EMS) (Sigma) before treatment with the selective agent, menadione. EMS diluted in serum-free medium was added to the medium of exponentially growing CHO-K1 cells to give a final concentration of 250μg/ml. Cells were incubated in the presence of EMS for 16 hours. The EMS-containing medium was then removed and the cells washed three times with PBS. Cells were then grown in drug free medium for 48 hours. This interval allows mutations to be expressed and represents approximately four cell doubling times. Cells were then exposed to 40μM menadione. This concentration of menadione was chosen as one likely to kill wild-type cells while allowing mutant cells of interest to survive (Jones and Sargent, 1974).

2.1.3 Cloning of menadione-resistant cell lines

Heterogeneous menadione-resistant cells were subsequently cloned by dilution in multiwell plates (Freshney, 1987).

2.1.4 Mycoplasma testing

Assays for mycoplasma were carried out routinely by the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh and were always found to be negative. The assay involved the growth of mycoplasma on defined agar plates and also a fluorescence test.

2.1.5 Assessment of cell sensitivity to cytotoxic compounds and heat

(a) MTT assay

Exponentially growing cells were harvested using trypsin/versene. A cell count was carried out using a hemocytometer (Scientific Furnishings). A suspension of either 5 x 10^3 cells/ml (for CHO cell lines) or of 1.5 x 10^4 (for EJ cell lines) was prepared by diluting the cell suspension with an appropriate volume of medium.

Aliquots of 180µl, which contained either 1 x 10^3 CHO cells or 3 x 10^3 EJ cells, were pipetted into the wells of a 96-well plate (Costar) using a multi-channel pipettor. Medium (200µl) only was added to the outermost wells. The plates were incubated overnight at 37°C, 5% CO_2 and 100% humidity to permit cell attachment. Test compounds were dissolved in ethanol (menadione), distilled water (bleomycin, menadione bisulphite, adriamycin, cadmium chloride, paraquat), PBS (sodium arsenite) and DMSO (ethacrynic acid) immediately before use and diluted in serum-free medium to give a range of concentrations. Drugs were added in a volume of 20µl to three or more microtitre wells. Medium only was added to six control wells. After three days, during which the microtitre plates were incubated at 37°C, 50µl of MTT (2mg/ml) was added to each well. The cells were incubated in the presence of MTT for 4 hours. The MTT/medium mixture was carefully aspirated from the wells and the formazan crystals dissolved in 50µl DMSO. Absorbance was measured at 540nm using a 2550 EIA reader (Biorad Laboratories). The first row of wells (to which only medium had been added) were used to zero the machine. The absorbance value for the control wells corresponded to 100% cell viability. The percentage viability of cells treated with different drug concentrations was calculated relative to this value. The mean and standard deviation were calculated for triplicate (or more) determinations. The LD_{50} for each compound was calculated from the plot of cell viability against drug concentration.

(b) MTT assay to assess resistance to heat

An adaptation of the MTT assay was used to assess resistance of cell lines to heat. Cells were seeded into 96 well plates at the following densities: 5 x 10^3 cells/ml (for CHO cell lines) or 1.5 x 10^4 (for EJ cell lines). They were allowed to adhere overnight. The following day the plates were placed in an incubator which had been allowed to equilibrate at 42°C. One plate was used for each time point. A plate was removed at each of the time points (15, 30, 45, 60, 90, 120, 150 and 180 minutes) and returned to an incubator at 37°C for three days. The MTT assay was then carried out as described above.

(c) Clonogenic assay

Cells were seeded into eight 25cm^3 culture flasks at a density 4 x 10^4 cells/ml. They were allowed to adhere for 24 hours. The medium was removed and replaced with fresh medium containing drug. Seven flasks were treated with drug in a range of concentrations. One flask was treated with drug-free medium and used as a control. Cells were incubated in the presence of drug for four hours. The growth

medium was then removed, the cells washed three times with PBS and harvested using trypsin/versene. Cells were centrifuged at 1800g and washed with medium twice before being resuspended in medium. Cells counts were carried out. Cells were diluted and then seeded into 6cm petri dishes containing 5ml of medium. Cells were distributed uniformly by gently sliding the dishes back and forth in one direction, and then applying the same motion in the orthogonal direction. Triplicate plates were prepared for each cell line at each drug concentration. The higher the drug concentration that had been used, the greater the number of cells seeded. The optimum number of colonies was fifty per plate. As well as being laborious to count, a greater number of colonies than this leads to inaccuracies because adjacent colonies become confluent. Small colony numbers lead to statistical inaccuracies. Cells were incubated at 37°C. During incubation petri dishes were disturbed as little as possible in order to minimise dislodgement of cells. After ten days the medium was removed and cells were washed twice with PBS. Adherent cells were fixed in a solution of acetic acid and methanol 1:3 (v/v), stained with 0.1% (w/v) crystal violet and counted under magnification. Only cell aggregates of 50 or more cells were regarded as colonies.

2.1.6 Generation of radiation survival curves

Radiation survival curves were generated for CHO-K1 and CHO-MRc40 cell lines. Cells were seeded into 25cm^3 culture flasks in 5 ml of media at a density of 4 x 10^4 cells/ml and allowed to adhere for 24 hours prior to irradiation. Cells were exposed to ^{60}cobalt gamma radiation at room temperature and in the presence of medium which had been conditioned for 24 hours. At the time of irradiation cultures were in log-phase growth. Irradiation was carried out under both oxic and hypoxic conditions. Hypoxic conditions were achieved by bubbling nitrogen gas through the growth medium for 30 minutes prior to irradiation. A dose range of 0 to 8 Gy was used for generation of oxic survival curves and a range of 0 to 16 Gy for hypoxic survival curves. Duplicate flasks were irradiated at the two highest dose levels. Control flasks were also duplicated.

Cell survival was measured using a clonogenic assay. Single cell suspensions were obtained from control flasks and from each of the irradiated flasks. An estimate of the number of cells required to give approximately 50 colonies was made, based on the expected survival fraction for each radiation dose. The cell suspensions were diluted appropriately and cells were seeded into triplicate 6cm petri dishes in 5ml of media. The plating efficiency was 85% for CHO-K1 and 76% for CHO-MRc40

(these percentages represent the mean of three experiments). The cells were incubated at 37°C for ten days and colonies were then counted.

Analysis of the radiation survival data was made using the software package "statworks". It gave a non-linear least squares fit of the data to the linear quadratic equation. This gave fitted curves for each data set individually. The combined data and curves were displayed using the mathematical software package "mathematica".

2.1.7 Cinemicroscopy

This work was carried out at the videomicroscopy and microinjection laboratory, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London. Cells were seeded at a density of 1 x 10^4/ml in 6cm petri dishes. Phase-contrast IMT-2 Olympus microscopes fitted with Sony CCD cameras were used to film the cells over several days. During filming cells were maintained in a perspex environmental chamber fitted to the microscopes in which temperature and carbon dioxide concentration could be controlled. Recordings were made using Betacam format video tape and then downloaded onto super VHS tape. A video printer was used to produce video stills. In order to measure intermitotic times, individual cells were followed on video and the time taken between cell divisions noted.

2.1.8 Electron microscopy

Cells were fixed with 1.5% glutaraldehyde in cacodylate buffer for 30 minutes, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Agar 100. The electron micrographs were carried out by Dr. D. Harrison, Department of Pathology, University of Edinburgh.

2.2 PREPARATION OF CELLULAR FRACTIONS

Cultures of exponentially growing cells were harvested from 75cm^3 flasks with trypsin/versene. Cells were washed three times in medium and resuspended in 400µl of sterile PBS. Cells were disrupted using three 5-second pulses from an MSE Soniprep 150 at maximal power. Each sample was cooled for 30 seconds on ice between each pulse of sonication. The sonicates were centrifuged at 13000g for 20 minutes in an Eppendorf centrifuge and the supernatant decanted. The supernatant (cytosolic fraction) and the pellet (cell membrane fraction) were stored at -70°C.

2.3 PROTEIN ESTIMATION

The Lowry (Folin-Ciocalteau) method was used to determine the protein content of cell samples (Lowry *et al*, 1951). Bovine serum albumin fraction V (Sigma) was used as a standard and the following reagents were used to carry out the assay:

(a) 70mM $NaCO_3 10H_2O$: 40mM NaOH

(b) 40mM $CuSO_4.5H_2O$

(c) 71mM Na K tartrate

(d) Stock Folin Ciocalteau reagent diluted 1:2 (v/v) with distilled water

(e) Alkaline copper solution which was made up fresh by adding 0.5ml of both solutions (b) and (c) and making the volume up to 50ml with solution (a).

Samples were diluted 1:20 (v/v) with 0.1M NaOH (giving a final volume of 1ml) and 5ml of solution (e) were then added. This solution was vortexed. After 10 minutes 0.5ml of solution (d) was added, the samples vortexed again and left for 45 minutes. Standard solutions of bovine serum albumin at 0, 25, 50, 80, 100, 120, 150 and 200 mg/ml were treated in the same way. The absorbance of the standard solutions were measured at 600nm on a Schimadzu UV 160 spectrophotometer and a standard curve generated. The amount of protein in each sample was then measured and determined at 600nm. A new standard curve was generated each time the protein content of cell samples was carried out. Standard protein concentrations were measured in duplicate and sample protein determinations in triplicate. For samples in which the protein concentration was particularly low a microassay was performed. For this the method described above was used but the volume of sample and solutions was scaled down by a factor of five.

2.4 ANALYSIS OF PROTEINS

2.4.1 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Two spacers were used to separate two glass plates which measured 12 x 12cm and 12 x 14cm and which had been cleaned with alcohol. The plates and spacers were held in place by two clamps from the Biorad Protean 1 apparatus (Biorad Laboratories). They were positioned on the gel-kit base ready for casting of the gel. A 12% (w/v) separating gel was prepared by mixing the following: 9.25ml separating gel buffer (1.5M Tris-HCl and 0.5%(w/v)

SDS, pH 8.8), 7.7ml of 2% (w/v) N,N′-methylene bisacrylamide, 10.7ml 40% (w/v) acrylamide, and 7.7ml distilled water). To this were added 2ml of 1% (w/v) ammonium persulphate solution (APS) followed by 20µl of N,N,N',N′-tetramethylene diamine (TEMED) to polymerise the gel. The gel solution was poured between the glass plates to a height of 12cm. A small amount of saturated butanol was pipetted between the plates to overlay the upper edge of the gel, ensuring that it was perfectly level. Polymerisation took approximately 30 minutes.

The stacking gel (4.5%) was prepared by mixing 1.1ml of 40% acrylamide, 0.7ml of 2% bisacrylamide, 2.5ml of stacking gel buffer (0.5M Tris-HCl, 0.5% (w/v) SDS, pH 6.8) and 5.4ml distilled water. To this were added 0.3ml of 1% APS and 10µl of TEMED. The saturated butanol was removed and the stacking gel mixture was poured to the top of the plates. A 15 or 20 track comb was slotted between the plates. Thirty minutes were allowed for polymerisation. The comb was gently removed. The gel was then slotted onto the upper reservoir of the Biorad Protean 1 kit which also contained the cooling system. The lower reservoir was filled with 1 litre of electrode buffer (0.52M Tris-HCl, 0.53M glycine, 35mM SDS, pH 7.3) diluted 1:10 (v/v) with distilled water. The upper reservoir was filled with electrode buffer which was also used to flush the wells of the stacking gel.

The protein samples were prepared by diluting them with distilled water and adding an equal volume of boiling mix which consisted of 10% (v/v) stacking gel buffer, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue. The final protein concentration was 1mg/ml. Samples treated in this way were heated to 100°C for 5 minutes prior to loading in order to denature and solubilize the proteins. The samples were loaded with a Hamilton syringe (Hamilton, Bonadoz, Switzerland). The upper reservoir with the gels attached were inserted in the tank (the lower reservoir). The cooling system was connected to provide a steady flow of cold water and the lid of the apparatus, housing the electrodes, was positioned. The current was maintained at 20mA per gel as the dye front ran through the stacking gel and was increased to 30mA per gel as the dye front ran through the separating gel.

2.4.2 Coomasie Blue staining of SDS-PAGE

To visualise the protein pattern after electrophoresis, gels were removed from the glass plate sandwich by prising the plates apart. The stacking gel was removed and the separating gel immersed in 0.25% (w/v) solution of Coomasie Brilliant Blue in water/methanol/acetic acid solution in a ratio of 5:50:7 by volume and allowed to

stain for 1 hour. Gels were destained in 5% (v/v) methanol and 7% (v/v) acetic acid. The destain solution was changed several times and the protein pattern viewed on a light box. If a permanent record was required the gel was photographed.

2.4.3 Western blot analysis

Western blot analysis was performed according to the method of Towbin *et al* (1979) and modified according to Adams *et al* (1985). Cytosolic proteins (30µg per track) were separated on 12% (w/v) SDS-PAGE gels. Standard, affinity purified protein was also loaded. After removal of the stacking gel, the separating gel was trimmed by cutting along the dye front. If more than one gel was being analysed, the corners of the gels were cut to permit later identification. The gel was transferred to 0.45mm nitrocellulose filter in a transblot cell containing buffer (2mM disodium ortho-phosphate, 20% methanol) for 12 hours at 250mA. After transfer the filter was blocked in 10% Bovine Serum Albumin (fraction V) and 0.5% Tween 20 in TBS for 1 hour. After blocking, the primary antisera was applied (at 1:500 dilution in 1% BSA and 0.05% Tween in TBS) for 1 hour. The primary antibodies used were raised in rabbits against the rat pi (acidic) class GST, human alpha (basic) class GST, human mu (neutral) class GST and rat Cu/Zn superoxide dismutase and in sheep against bovine catalase (Serotec). The filters were then washed over 25 minutes (two 5 minute washes with TBS, two 5 minute washes with TBS and 0.1% Tween and one 5 minute wash with TBS). The second antisera (HRP-conjugated with anti-rabbit IgG or HRP-conjugated with anti-sheep IgG) in 1% BSA in TBS was applied for 1 hour at a dilution of 1:3000. The filters were then washed as before. Antibodies were used four times and then discarded. Bands were visualised using one of the following methods:

(a) Peroxidase staining using 4-chloro-1-naphthol as substrate

Filters were incubated at room temperature in 200ml of TBS containing 3.4M 4-chloro-1-naphthol (Sigma) and 80µl of 30% (v/v) hydrogen peroxide until the bands became visible. The reaction was terminated by placing the filters in distilled water.

(b) Autoradiography using ^{125}I protein A

Bound antibody was visualised by the addition of 0.19 MBq of ^{125}I-conjugated protein A (Amersham Int. Ltd.) to the filter in 50ml TBST. After incubation for an hour, the iodine was removed and the filter repeatedly washed in TBST. The filter was air dried, covered in saranwrap and exposed to autoradiograph film in an X-ray cassette with intensifying screens. The cassette was stored at -70°C until the

autoradiograph was developed.

2.4.4 Short-term labelling of proteins with ^{35}S-methionine

CHO-K1 and CHO-MRc40 cells were grown in 3cm plastic petri dishes to 70% confluency. The medium was removed and retained. The cells were treated with menadione or sodium arsenite. Both drugs were added to the cells in PBS and the concentrations of menadione used were 20, 30, 40 and 50μM and the concentrations of sodium arsenite were 10, 20, 50, 250μM. A control dish for each cell line was treated with PBS alone. After 30 minutes the drug was removed, the cells washed twice with PBS and the conditioned medium replaced. After 1 hour the medium was removed and replaced with 1.0ml of α-MEM without methionine (Gibco) supplemented with glutamine and 10% foetal calf serum for 30 minutes to deplete intracellular pools of methionine. This was then replaced with methionine-deficient medium containing 10mCi ^{35}S-methionine (>800Ci/mMol, Amersham Int. Ltd.) for 30 minutes at 37°C. The radioactive medium was removed, cells were washed twice with ice-cold PBS and then lysed by adding 0.2ml of SDS sample buffer (2% sodium dodecylsulphate, 10% glycerol, 3% β-mercaptoethanol, 50mM Tris/HCl (pH 6.8), 0.1mM phenylmethylsulphonyl fluoride and 0.01% bromophenol blue). The lysate was sonicated for 10 seconds and then heated to 100°C for 2 minutes. Samples were loaded on a 12% SDS-PAGE gel on the basis of equivalent cell number and electrophoresis carried out. The gel was fixed in 7% acetic acid for 30 minutes and then 7% acetic acid/5% glycerol for 30 minutes. The gel was then dried under vacuum for 2 hours at 75°C on a gel drier.

2.4.5 Two-dimensional gel electrophoresis

The method used for 2-dimensional gel electrophoresis was that of O'Farrell *et al* (1977) with modifications. Glass tubes (14cm in length and 2mm inside diameter) were soaked overnight in a 50-50 mixture of concentrated nitric and concentrated sulphuric acid. They were rinsed thoroughly until the pH of the water used to rinse them was 7.0. The tubes were dried and marked at 12cm from the bottom. The bottom was sealed by wrapping with parafilm. The tubes were then placed standing up in a rack. Isoelectric focusing (IEF) acrylamide gel was prepared and contained 5.5g urea (BDH), 1.33ml of 28.38% (w/v) acrylamide/1.62% (w/v) N,N'-methylene-bis-acrylamide, 2ml NP-40 (10%), 2ml distilled water and 500μl ampholines (Pharmacia) (400μl pH 3.5-10 and 100μl pH 5-7). The IEF acrylamide gel mix was

heated at 37°C until the urea had dissolved and was degassed for 10 minutes. 10µl each of 10% ammonium persulphate and TEMED were added. The gel mixture was immediately loaded into the glass tubes using a 5ml syringe fitted with a narrow metal needle. The tip of the needle was inserted to the bottom of the tube which was then slowly filled (to the 12cm mark) to prevent entrapment of bubbles. The gel mix was overlaid with distilled water and left to polymerise for 45 minutes.

The electrode solution for the lower chamber of the first dimension was prepared and consisted of 10mM phosphoric acid (0.575ml phosphoric acid in 500ml of degassed distilled water). Electrode solution for the upper chamber consisted of 20mM NaOH (400mg NaOH in 500ml of degassed distilled water). The glass tubes were taken from the rack and the parafilm removed using a scalpel. Excess liquid was removed by shaking. The tubes were inserted into the lower chamber. Cell lysate samples were diluted in IEF sample buffer (2% (w/v) NP-40, 2% (w/v) ampholines, 5% (w/v) β-mercaptoethanol and 9.5M urea). Samples (up to 50µl in sample buffer) were loaded into the top of the tubes using a Hamilton syringe followed by 40µl of overlay buffer (IEF sample buffer diluted 1:3 with distilled water). The tubes and upper chamber were filled with upper chamber buffer. The gels were connected to the power pack and run at constant power. Initially the current was adjusted to give a voltage of 100V, and was limiting. As electrophoresis progressed, the voltage rose until it became limiting at 400V. The ampage was maintained at the initial value, until a voltage of 400V was reached. Electrophoresis was continued for 10 hours at 400V and then for one hour at 800V.

At the end of the run, the glass tubes were removed from the electrophoresis apparatus. A short needle was used to loosen the gels at both ends of the glass tubes. Gels were extruded from the tubes with the aid of air pressure delivered by a syringe. Prior to second dimension electrophoresis gels were equilibrated in SDS sample buffer and then stained using Coomasie Blue. 12% separation gels were prepared (as described in section 2.4.1) and allowed to polymerise for 1 hour. A small amount of low melting point agarose (1% in one quarter strength stacking buffer) was used to make a fixed contact between the stick gel and the SDS-PAGE gel. The stick gel was laid on the agarose while still molten making sure there were no bubbles between the two. Electrophoresis was carried out at 15mA through the stacking gel and 30mA through the separating gel until the bromophenol blue tracking dye reached the bottom of the gel. The proteins were then detected by Coomasie Blue or silver nitrate. If radiolabelled samples had been used the gels were fixed, dried under vacuum and exposed to radiograph film at room temperature.

2.4.6 Silver staining of polyacrylamide gels

A silver stain kit (Amersham, RPN.17) was used according to the manufacturers instructions.

2.5 ISOLATION AND ANALYSIS OF NUCLEIC ACIDS

2.5.1 Isolation of RNA

The single-step guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) was used. This method employs the powerful protein denaturant guanidinium hydrochloride to destroy ribonuclease activity and depends on the differential precipitation of RNA and DNA at -20°C followed by centrifugation at 10,000g. To minimise degradation of RNA by contaminating nucleases, Eppendorfs and pipette tips were soaked in distilled water containing 0.1% DEPC for 12 hours before autoclaving. The denaturing solution consisted of 4M guanidinium thiocyanate (Fluka), 25mM sodium citrate (pH 7.0), 0.5% sarcosyl (sodium lauroyl sarcosinate, 30% w/v) and 0.1M β-mercaptoethanol. 1.7ml of denaturing solution was used per $75cm^3$ culture flask. The denatured cell mixture was transferred to a 15ml polypropylene tube (Falcon). Sequentially, 0.17ml of 2M sodium acetate (pH 4.0), 1.7 ml of water-saturated phenol (nucleic acid grade) and 0.34ml of chloroform-isoamyl alcohol mixture (49:1) were added to the cell lysate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. Samples were then centrifuged at 10,000g for 20 minutes at 4°C. After centrifugation RNA was present in the aqueous phase and DNA and protein in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1.7ml of isopropanol and then placed at -20°C for at least 1 hour to precipitate RNA. Sedimentation at 10,000g for 20 minutes was again performed and the resulting RNA pellet was dissolved in 0.52ml of denaturing solution, transferred to a 1.5ml Eppendorf tube and precipitated with an equal volume of isopropanol for 1 hour at -20°C. After centrifugation in an Eppendorf centrifuge for 10 minutes at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented and air dried. The final pellet was dissolved in 50µl of 0.5% SDS at 65°C for 10 minutes and stored at -70°C.

2.5.2 Spectrophotometric quantification of DNA and RNA

DNA and RNA concentrations were measured spectrophotometrically at 260nm and 280nm. Concentrations of nucleic acid given below correspond to a change of 1 absorbance unit.

1 absorbance unit at 260nm = 50mg/ml for double stranded DNA and RNA

1 absorbance unit at 260nm = 40mg/ml for single stranded DNA and RNA

The ratio between absorbance at 260nm and 280nm provides an estimate of the purity of the preparation. For a pure DNA preparation the Absorbance at 260nm/Absorbance at 280nm ratio was 1.8, and for RNA it was 2.0.

2.5.3 Northern blot analysis

For northern blot analysis, a horizontal 1.5% agarose gel in MOPS buffer (0.2M MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0) containing 18% formaldehyde solution from a stock formaldehyde solution of 40% (v/v) was prepared. A 15µg aliquot of RNA was mixed with 3 volumes of sample buffer (0.1M MOPS, 25mM NaOAc and 5mM EDTA, pH 7.0) and heated to 50°C for 30 minutes. A 3µl aliquot of RNA ladder was treated in the same way as the samples. Loading dye consisting of 50% glycerol (v/v), 0.4% xylene cyanol (w/v) and 0.4% bromophenol blue (w/v) was added at one-tenth volume prior to loading. The RNA samples were electrophoresed at 45V for 15-20 hours in MOPS buffer, using a BRL horizontal electrophoresis kit, model 41 (BRL). The RNA was transferred by capillary action in 10 x SSC (1.5M sodium chloride, 0.15M trisodium citrate) to hybond paper as described by Southern (1975). The hybond filter was rinsed in 2 x SSC (0.3M sodium chloride, 0.03M tri-sodium citrate), air dried and then irradiated with UV from a short wavelength transilluminator for 2.5 minutes. The part of the filter corresponding to the lane containing the RNA ladder was cut from the rest of the filter. The strip was stained with methylene blue and then destained in water until the RNA bands were visible. This was retained and used to assess the size of bands on the northern blot. The filter was prehybridised in 20ml of hybridisation mix consisting of the following: 1% SDS, 50% formamide, 1M NaCl (in SSC), 10% dextran sulphate and 100µg of denatured salmon sperm for four hours at 42°C in a Techne oven. The radiolabelled cDNA probe was added and hybridisation continued overnight at 42°C. The filter was washed twice in 2 X SSC (for 10 minutes each) at room temperature, then twice in 2 X SSC/1%SDS at 65°C (for 30 minutes each) and finally twice in 0.1 X SSC at room temperature (for 30 minutes each). The filter was

air dried, wrapped in parafilm and autoradiographed using kodak Xar5 film. To strip radioactivity from filters prior to re-probing they were placed in a solution consisting of 5mM Tris-HCl, 2mM EDTA and 0.1% Denhardts and heated to 65°C for 1 hour. The stripping solution was changed twice and washing continued for 30 minutes at 65°C following each change. Filters were then covered in parafilm and exposed to autoradiograph film overnight. If all radioactivity had been satisfactorily removed from the filter, hybridisation with a second cDNA probe was carried out.

2.5.4 Random priming of isolated cDNA with radio-labelled ^{32}P αdCTP

The cDNA to be labelled was isolated by electrophoresis on a low melting point agarose gel. The relevant band was cut from the gel, placed in a 1.5ml Eppendorf and 1.5ml of distilled water was added for every gram of agarose. This mixture was heated to 100°C for 10 minutes. An aliquot (containing approximately 90ng DNA) was removed for labelling and the remainder stored for future use at -20°C. Radiolabelling was carried out according to the method of Feinberg and Vogelstein (1983). The following were added to the DNA sequentially: 5µl of oligolabelling buffer, 2µl of BSA (1mg/ml), 18µl distilled water, 50mCi of ^{32}PαdCTP and 1 unit of Klenow enzyme. Oligolabelling buffer was prepared according to the method of Maniatis *et al* (1982). The labelling reaction was allowed to proceed overnight at room temperature. The amount of radioactivity incorporated into the DNA was estimated by DEAE-cellulose paper chromatography. A 1µl aliquot of the labelling reaction mixture was pipetted onto a strip of DEAE-cellulose paper close to its lower edge and the chromatograph was run for 30 minutes in 0.3M ammonium formate (pH 8.0). The DEAE-cellulose paper was air dried, wrapped in parafilm and exposed to autoradiograph film for 30 minutes. Since DNA does not move up the chromatography paper, any DNA-incorporated radioactivity remained at the lower edge of the paper.

2.5.5 cDNA probes

The DNA probe for GST-Pi was derived from the 0.8kb *Eco*R1 fragment of human GST-Pi cDNA (Kano *et al*, 1987). The DNA probe for glutathione peroxidase was derived from the 0.7kb *Eco*R1 fragment of the mouse glutathione peroxidase cDNA (Chambers *et al*, 1986). An 804 base-pair fragment corresponding to nucleotides 892 to 1695 of the published sequence (Gipp *et al*, 1992) of γ-GCS was obtained from human skin fibroblast DNA using the polymerase chain reaction.

A 343 base-pair fragment corresponding to nucleotides 1199 to 1541 of the published sequence of human glutathione reductase (Tutic *et al*, 1990) was obtained from the same source. The DNA probe for HO was derived from the 1.0kb *Eco*R1 fragment of heme oxygenase cDNA (Keyse and Tyrrell, 1989). Northern blots were reprobed with the *Pst* I fragment of the rat glyceraldehyde phosphate dehydrogenase (GAPDH) gene as a loading control (Piechaczyk *et al*, 1984). DNA probes for northern analysis were labelled by random prime incorporation.

2.5.6 Gel electrophoresis of DNA

The running buffer used was either TBE (89mM Tris-HCl, 89mM boric acid and 2 mM EDTA) or TAE (0.4 mM Tris-HCl pH 8.2, 0.2M sodium acetate and 10 mM EDTA). The gel was composed of the appropriate running buffer and 1% agarose. A higher concentration of agarose was used for the resolution of small fragments. Electrophoresis was carried out at 20-25V. A 1µl aliquot of 1-kb ladder was run on the gel to allow accurate assessment of DNA fragment size. After electrophoresis the gels were stained in distilled water containing 0.025% ethidium bromide, viewed on a UV transilluminator and photographed.

2.5.7 Restriction endonuclease digest

Restriction enzymes were purchased from Boeringer-Mannheim, New England Biolabs, BRL and Amersham International. Reaction mixtures were set up according to the suppliers instructions and according to the requirements of the individual enzyme. A typical digest reaction mixture would contain 20µl of the DNA to be digested, 3µl of RNAase (10mg/ml), 10units/µl of restriction enzyme, 4µl of reaction buffer and 10µl of distilled water. The reaction buffer was selected according to which enzyme was being used and was usually supplied by the manufacturer at 10 times the required concentration. This mixture was incubated at 37°C for 2 hours or overnight. After digestion the reaction was stopped by the addition of 4µl of loading dye (20% ficoll, 10mM EDTA and bromophenol blue). DNA fragments were then analysed by agarose gel electrophoresis.

2.5.8 The polymerase chain reaction

This technique was used to generate the γ-GCS and GR DNA probes. For a 100µl reaction mix the following were added; 1µl of the DNA template (1 to 3ng DNA), 1µl of each oligonucleotide (1mg/ml), 8µl of dNTPs (2.5mM), 10µl of

promega reaction buffer (10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM magnesium chloride and 0.01% gelatin) and 0.5µl of DNA Taq 1 DNA polymerase 4U/ml (Promega). The volume was made up to 100µl with distilled water and overlayed with mineral oil after mixing. A Techne PCR machine was programmed to carry out 30 cycles of PCR:

1) 5 minutes at 92°C
2) a. Melting: 92°C for 30 seconds
   b. Annealing: 50°C for 45 seconds
   c. Extension: 72°C for 60 seconds
3) 5 minutes at 72°C

Part 2) was repeated 30 times and was responsible for the amplification of the DNA. The efficiency of the reaction was assessed by running a 10µl aliquot on an agarose gel.

2.6 PLASMID CLONING

2.6.1 Plasmid preparations

(a) Alkaline lysis

A bacterial culture was grown overnight at 37°C with shaking in L-Broth (L-Broth contained 10g bacto-tryptone (Difco), 5g yeast extract (Difco) and 5g sodium chloride for 1 litre). The overnight culture was centrifuged at 2000g and the cell pellet then resuspended in 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA, 2mg/ml lysozyme (Sigma) and after 30 minutes 1%SDS and 0.2M NaOH were added to disrupt the cells. The bacterial genomic DNA and cell debris were precipitated with isopropanol. The plasmid was further purified by a phenol/chloroform extraction, which was followed by ethanol precipitation or for large cultures by density gradient centrifugation through caesium chloride (Boehringer-Mannheim). The purified plasmids were resuspended in TE buffer (10mM Tris-HCl, pH 8.0, and 1mM EDTA) and stored at -20°C. Purification of the plasmid was confirmed by analysis on an agarose gel. For constructs, an appropriate restriction digest was performed and the DNA then run out on an agarose gel to demonstrate the presence of the insert.

(b) Quiagen columns

Quiagen columns is the trade name of a product used for rapid purification of high quality plasmid DNA. The procedure is similar to that described above but

carried out with buffers supplied by the manufacturer. Following removal of bacterial genomic DNA the solution is passed over a DNA affinity resin to which the plasmid DNA binds. This is then eluted with a high salt buffer and isopropanol in the final step.

2.6.2 Plasmid cloning

(a) Preparation of vector and insert DNA for cloning

Plasmid DNA was prepared by restriction endonuclease digestion to generate compatible ends for cloning. Insert DNA was prepared by the Imperial Cancer Research Fund central laboratory. Electrophoresis of a sample of DNA (0.3µg) was carried out to check that the digestion reaction had gone to completion. The following were added to the remainder of the digested DNA: 1µl of calf intestinal alkaline phosphatase (CIAP) (Promega), 10µl CIAP 10 X buffer and distilled water to a give a final volume of 100µl. This reaction, which removes 5' phosphate groups and thus prevents recircularisation of the vector during ligation, was allowed to proceed for 1 hour at 37°C. To remove CIAP from the reaction mixture (which may interfere with ligation of the phosphatase-treated vector to the insert DNA) one volume of TE-saturated phenol/chloroform was added to the reaction mixture. This was vortexed and then centrifuged at 13,000g for 2 minutes. The aqueous phase was transferred to a new Eppendorf and extraction with chloroform:isoamyl carried out. This step was repeated and then 0.5 volume of 7.5M ammonium acetate was added to the aqueous phase. Two volumes of ethanol were added and the sample stored at -70°C for 30 minutes. The DNA pellet was collected by centrifugation at 12,000g for 2 minutes. The supernatant was discarded, the pellet washed with 1ml of 70% ethanol and dried briefly in a vacuum dessicator. The pellet was resuspended in 15-20µl of distilled water. The DNA concentration was then measured by spectroscopy.

(b) Ligation of vector and insert DNA

Plasmid and insert DNA were placed in 1.5ml Eppendorfs in a ratio of 1:5 or 1:10. The following were added sequentially: 1µl of T_4DNA ligase (Boehringer-Mannheim), 2µl of 10 X ligase buffer, 1µl of 10mM ATP and 20µl of distilled water. The reaction was allowed to proceed overnight at room temperature. Following this reaction the plasmid DNA was transformed into competent bacterial cells (see below).

2.6.3 Preparation of competent *E. coli* cells

An overnight culture of the appropriate strain was prepared. A 1ml aliquot of this culture was added to 100ml of pre-warmed L-Broth. This was incubated with shaking at 37°C for approximately 2 hours until the absorbance of the culture at 600nm was between 0.2 and 0.4. The culture was transferred to 50ml Falcon tubes, placed on ice for 15 minutes and then centrifuged (in a cold room) for 10 minutes at 4000g. The cell pellet were resuspended in 75mM calcium chloride and incubated on ice for 20 minutes. After centrifugation at 4°C, the cells were resuspended in 4 ml of the following solution: 10mM MOPS (pH 7.0), 75mM calcium chloride, 10mM rubidium chloride and 15% glycerol. All the components of this solution other than MOPS was autoclaved and the final solution was filter sterilised. Cells were kept on ice for 20 minutes prior to use or were snap frozen in liquid nitrogen and stored in small aliquots at -70°C. The bacterial strain used was *E. coli* JM109 e14 (mcr A), recA1, end A1, gyr A96, thi-1.

2.6.4 Transformation of *E. coli*

Competent *E. coli* cells were prepared as described above. The plasmid to be transformed (10 to 20µg of DNA) was added to 50µl of the following solution: 10mM Tris-HCl (pH 8.0), 10mM magnesium chloride, and 10mM calcium chloride. This was added to 200µl of competent cells, mixed by inversion and incubated on ice for 30 minutes. The mixture was then incubated at 45°C for 5 minutes. L-Broth (1ml) was added and the mixture incubated for 30 minutes at 37°C. The cells were then centrifuged to form a pellet. The pellet was resuspended in residual media and the cells plated onto L-Broth agar (15g L-Agar per litre L-Broth) containing the appropriate selectable antibiotic (ampicillin). Plates were inverted and incubated at 37°C overnight. The bacteria which were successfully transfected with plasmid containing insert formed colonies.

2.6.5 Screening bacteria for the presence of recombinant plasmids

The method of Grunstein and Hogness (1975) was used to screen large numbers of bacteria for transfectants containing plasmid with the desired insert. Individual colonies were transferred (using sterile toothpicks) in duplicate onto nitrocellulose filters marked with a grid (Schleicher and Schuell) which had been placed on agar plates containing ampicillin, the selective antibiotic. Positive and negative controls were also streaked out onto both filters. Following incubation overnight at 37°C,

both filters were marked to allow future orientation. One plate was designated the master plate, sealed and stored inverted at 4°C. The other filter was placed colony side up, on 3MM paper soaked in 10% SDS for 3 minutes, denaturing solution (1.5M sodium chloride, 0.5N sodium hydroxide) for 5 minutes and neutralising solution (0.5M Tris-HCl, pH 7.5, 1.5M sodium chloride) for 5 minutes. The filters were dried, colony side up, on tissue paper for 30 to 60 minutes and baked for 1 hour at 80°C.

The filters were washed to remove bacterial debris in 50mM Tris-HCl (pH 8.0), 1.5mM EDTA and 1% SDS at 42°C. Filters were then incubated at 65°C for 2 hours in 25ml of the following hybridisation solution: 6 x SSC (1 x SSC is 150mM sodium chloride and 15mM sodium citrate), 2 x Denhardts (1 x Denhardts consists of ficoll, polyvinylpyrrolidine and bovine serum albumin all at 1mg/ml), 0.5% SDS and 0.05% sodium pyrophosphate. The radiolabelled probe, corresponding to the insert, was heated to 100°C for 5 minutes and then added to the hybridisation mix. The filters were hybridised overnight, washed with 3 to 4 changes of 2 x SSC and 0.1% SDS at room temperature for 5 minutes and then washed in 1 x SSC and 0.1% SDS for 30 minutes at 65°C. The filters were air dried on 3MM paper, covered with saran wrap and exposed to autoradiography film overnight in a cassette with intensifying screens at -70°C. Colonies in which the vector contained an insert gave a strong signal.

2.7 SEQUENCING OF DNA

2.7.1 Preparation of single-stranded DNA for sequencing

Sequenase version 2 (United States Biochemical) which is a modification of the chain termination method of Sanger (1977) was used according to the manufacturer's instructions.

2.7.2 DNA sequencing gel electrophoresis

Sequencing gel plates were cleaned with detergent and water and then wiped with distilled water and ethanol. One plate (that with electrodes) was siliconised using Replocote. Spacers were cleaned with water and ethanol and placed flush to the edge of the siliconised surface. The other gel plate was placed on top and the sandwich lifted into a vertical position. The spacers were pushed down so that their lower edge was flush with that of the gel plates. Side clamps were used to hold the assembled plates in position. Four strips of 3MM paper were placed in the bottom of

the gel tank. A 12% gel mixture containing 16.8g of urea (BRL ultra pure grade), 4ml of 10 X TBE, 6ml of 40% acrylamide and 40ml distilled water was prepared. To this were added sequentially: 240µl 10% AMPS and 33µl TEMED. This gel mixture was poured onto the filter strips. The gel plates were placed in the tray, pushed down and the screws tightened. The gel was allowed to polymerise for 20 minutes. A 6% gel mixture was prepared and contained 7M urea, 7.5ml 10 X TBE, 11.25ml 40% acrylamide and 75ml distilled water. To this were added sequentially: 450µl 10% AMPS and 63µl TEMED. A 50ml syringe was filled with gel mix and the gel was injected between the plates with constant pressure. After polymerisation, radio-labelled DNA samples were loaded in adjacent wells on the gel. Following electrophoresis the gel was fixed in 10% acetic acid and 10% methanol, dried for 2 hours under vacuum and exposed to autoradiograph film at room temperature.

2.8 EXPRESSION OF CLONED GENES

2.8.1 Calcium phosphate technique for gene transfer

Exponentially growing cells were harvested by trypsinisation. Cells were reseeded at a density of 10^6 per 6cm diameter petri dish which contained 5 ml of pre-warmed fresh medium. They were incubated at 37°C for 24 hours. Donor DNA (0.5ml) was placed in a plastic bijou at a concentration of 80mg/ml in 0.1mM EDTA and 1.0mM Tris-HCl (pH 8.0). To this was added 0.4ml of 0.1mM EDTA, 1.0mM Tris-HCl (pH 8.0) and 0.1ml of 2.5M calcium chloride. The vials were vortexed. The DNA was slowly added (over about 30 seconds) with continuous mixing to 1.0ml of 2 x Hepes buffered saline (HBS) (0.28M NaCl, 50mM Hepes, 1.5mM $Na_2HPO_4.2H_2O$ and distilled water to 100ml) in a second vial. This was mixed immediately by vortexing and the solution was stored at room temperature for 30 minutes by which time a fine precipitate formed. This DNA-calcium phosphate suspension (0.5ml) was added to each cell culture flask containing 5ml growth medium. The flasks were incubated at 37°C for 24 hours to allow absorption of the DNA-calcium phosphate precipitate by the cells. The medium was replaced and incubated for a further 24 hours to allow expression of the transferred gene. The medium was changed and menadione, H_2O_2 or PMA was added for 20 hours prior to lysis of the cells for CAT assay.

2.8.2 Chloramphenicol acetyltransferase (CAT) assays

(a) Preparation of extracts

The medium was removed from the cells by gentle aspiration and the monolayer washed three times with PBS. The dishes were angled for 2 to 3 minutes to allow the last traces of PBS to drain to one side. These were removed by aspiration. PBS (1ml) was added to the dishes and using a cell-scraper the cells were harvested and then transferred to an Eppendorf. The samples were stored on ice until all samples had been collected. Cells were recovered by centrifugation and the PBS removed from the cell pellet by aspiration using a disposable pipette tip attached to a vacuum line. The cell pellet was then either stored at -20°C or analysed immediately.

(b) Thin-layer chromatography (TLC)

The cell pellet was resuspended in 100µl of 0.25M Tris-HCl (pH 7.8) and then vortexed vigorously. The cells were disrupted by three cycles of freezing in dry ice/ethanol and thawing at 37°C. The cell lysate was centrifuged at 13000g for 5 minutes at 4°C in an Eppendorf centrifuge. The supernatant was transferred to a fresh 1.5ml Eppendorf. A 50µl aliquot was removed for use in the CAT assay and the remainder was frozen at -20°C. The 50µl aliquot was incubated at 65°C to inactivate deacetylases. The CAT reaction mix consisted of 1ml of 1M Tris-HCl (pH 7.8), 200µl of ^{14}C-labelled chloramphenicol (60mCi/mmole, diluted in water to 0.1mCi/ml) (Amersham International) and acetyl coenzyme A (Sigma) (freshly prepared at a concentration of 3.5mg/ml in water). For each 50µl of cell extract to be tested 80µl of CAT reaction mixture was added. The mixture was incubated at 37°C for 1 hour. Ethyl acetate (1ml) was added and the samples were mixed thoroughly by vortexing for 10 seconds and then centrifuged for 5 minutes at room temperature at 13000g. The acetylated forms of chloramphenicol partition into the organic (upper) phase and unacetylated chloramphenicol remains in the aqueous phase. An aliquot (900µl) of the upper phase was transferred to a fresh tube. The ethyl acetate was evaporated under vacuum by placing the tubes in a rotating evaporator (Speedvac) for 1 hour. The reaction products were redissolved in 25µl of ethyl acetate. A 15µl aliquot of the dissolved reaction products was applied to the origin of a 25mm silica gel, TLC plate. A TLC chamber containing 200ml of chloroform:methanol (95:5) was prepared. The TLC plate was placed in the chamber which was then closed. The solvent front was allowed to move approximately three quarters of the way to the top of the plate. The plate was removed from the chamber and allowed to dry at room temperature. The plate was then exposed to autoradiograph film.

2.9 MEASUREMENT OF INTRACELLULAR THIOLS BY HPLC

2.9.1 Preparation of cells

Cells were grown to 75% confluency. Menadione was dissolved in ethanol to a stock concentration of 10^{-2}M. The appropriate amount of stock menadione solution was added to the cell culture medium to give a final concentration of 25μM. Menadione was added to one flask of each cell line at the following times before cells were harvested: 24, 12, 6, 3, 2, 1 hours and 20 minutes. A control flask for each cell line was treated in the same way as the other flasks except that menadione was omitted. Cells were harvested and washed twice in PBS. They were counted and a final cell suspension of 3×10^6 cells/ml PBS was prepared.

2.9.2 Derivatization of the sample

The method described by Cotgreave and Moldéus (1986) was used and is based on the sulphydryl-reactive agent monobromobimane (mBBr). This agent has high specificity and reactivity towards sulphydryls, with which it forms highly fluorescent adducts. The low molecular weigh thiol-bimane adducts are suitable for chromatographic separation. Identification and quantification of glutathione and cysteine was achieved by including standard mixtures of glutathione and cysteine in each HPLC run.

(a) Free thiols

This method results in the recovery of both free, reduced low molecular weight thiols and bromobimane accessible protein thiols as their corresponding bimane adducts. A 100μl aliquot of each cell suspension was transferred to an Eppendorf tube. Duplicate samples were prepared for both cell lines at each time point. 10μl of PBS and 100μl of 8mM mBBr in 50mM *N*-ethyl morpholine (pH 8.0) were added to the Eppendorfs. MBBr is relatively insoluble and so was predissolved in a small volume of acetonitrile (HPLC grade). The sample was stored at room temperature in the dark for 5 minutes and then acidified to stop the reaction by the addition of 10μl of 100% (w/v) trichloracetic acid. Precipitated protein was removed by centrifugation at 3000g for 5 minutes. Aliquots (100μl) of the derivatised samples were then transferred to glass vials prior to HPLC.

(b) Total thiols

MBBr will not react directly with disulphides and so this method includes prederivatization reduction with dithiothreitol (DTT) and so renders oxidised low molecular weight thiols and oxidised protein thiols accessible to derivatization with monobromobimane. The efficiency of release of thiols from protein is unknown. Thus an increased recovery would be expected due to reduction of both low molecular weight, soluble disulphides and disulphides between low molecular weight thiols and protein thiols. A 100µl aliquot of the cell lysate was treated with 100mM DTT, vortexed and allowed to stand for 30 minutes at room temperature and then derivatised by the addition of 20mM mBBr in 50mM *N*-ethyl morpholine (pH 8.0). The samples were then treated in the same way as those for the measurement of free thiols.

2.9.3 High Performance Liquid Chromatography

A Waters (Milford, Massachusetts) Novapak steel column (3.9 x 150mm) packed with 4mm octadodecyl silica reversed phase material was used for the chromatographic separation of thiol-monobromobimane derivatives. The column was protected by a Waters Guard-Pak precolumn packed with the same material. The chromatographic system consisted of two Model 410 pumps, an automated gradient controller, a Waters intelligent sampler processor model 710 (automatic injection system), and a data module (M730) for peak integration. A fluorescence detector (Model 420) was used for peak detection. The elution solvent A was 10% (v/v) HPLC grade methanol and 0.25% (v/v) glacial acetic acid in distilled water (pH 3.9). Solvent B was 90% (v/v) methanol and 0.25% (v/v) glacial acetic acid in distilled water (pH 3.9). Both buffers were degassed using a sonic bath before use. The water-methanol-acetic acid elution programme used in the chromatography was as described by Newton et al (1981) but modified as follows: 0-15 minutes, 3% buffer B, isocratic at 1ml/min; 15-17 minutes, 90% buffer B; 17-25 minutes, 3% buffer B (column regeneration). Standard solutions of cysteine and reduced glutathione (Sigma) were prepared with PBS (pH 7.4) in the same way as the test solutions. Every fifth position on the sample processor was taken by a vial containing standard thiol solution to ensure consistency throughout the HPLC run.

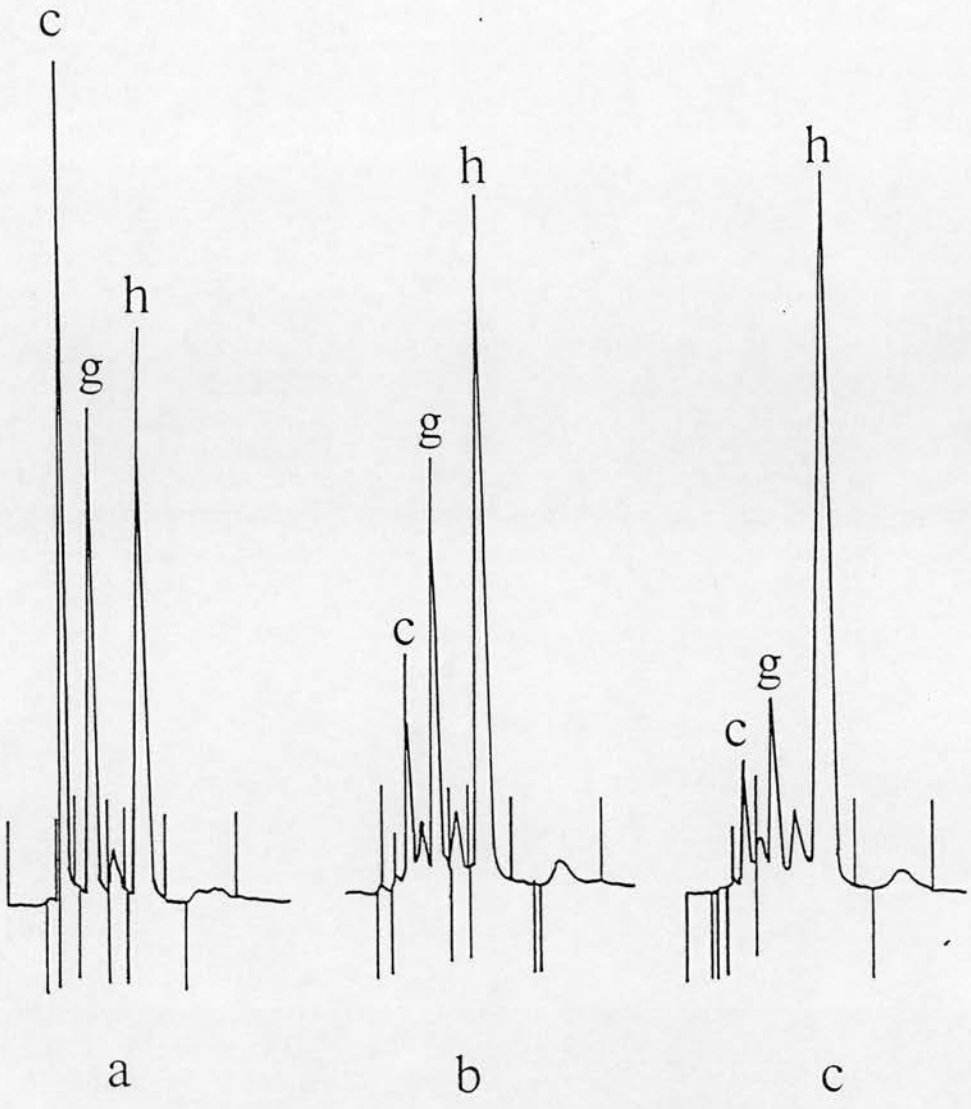


Figure 2.1 *HPLC traces of the separation of mBBr derivatives*

Some typical HPLC traces obtained from the analysis of cysteine and glutathione with mBBr.

Trace **(a)** obtained from derivatisation of a standard sample containing 1.245nmol and 1.202nmol of cysteine and glutathione respectively.

Trace **(b)** obtained from derivatisation of CHO-MRc40 cells (control sample)

Trace **(c)** from derivatisation of CHO-MRc40 cells 3 hours after the addition of 25μM menadione, showing depletion of both thiols compared with control (untreated) sample.

Peak c CySH-mBBr adduct (retention time, 3.0 minutes)

Peak g GSH-mBBr adduct (retention time, 4.5 minutes)

Peak h Reagent hydrolysis peak (retention time, 7.0 minutes)

2.10 ^{1}H SPIN ECHO NUCLEAR MAGNETIC RESONANCE OF WHOLE CELLS

2.10.1 Preparation of cells

Cells were harvested at 75% confluency and washed 3 times in $^{2}H_2O$/NaCl (0.154M) and transferred to a 5mm NMR tube. $^{2}H_2O$ was obtained from Goss Scientific Instruments Ltd. The cell concentration was 10^{7}cells/0.5ml.

2.10.2 Recording of NMR spectra

NMR spectra were recorded using a Carr-Purcell-Meiboom-Gill sequence $(90°\text{-}t\text{-}180°\text{-}t)_n$ with a delay time (t) of 60ms and one repetition (n=1) of the pulse sequence. A Buker 400MHz spectrometer was used to record all spectra. Samples were maintained at 20°C during data collection and the data from 2000 complete pulse sequences were accumulated for each spectrum. The free induction decay (FID) was collected in 8K of memory (acquisition time 0.64 seconds/scan) to which a 0.5Hz line-broadening function was applied, prior to zero filling to 64K Fourier transformation. The 90° pulse was generated with a 7.5ms pulse width. The water was eliminated from the spectrum by presaturation (55dB) during relaxation delay (2 seconds). Once a satisfactory control spectrum had been obtained, menadione was added to the NMR tube. Menadione bisulphite was used in some of the NMR experiments because it is more soluble in water than menadione. The ethanol required to dissolve menadione itself would have interfered with the spectra.

2.11 ENZYME ACTIVITY ASSAYS

2.11.1 Glutathione S-transferase assay

Assays of GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate were carried out in a Cobas Fara centrifugal analyser (Roche diagnostics, Welwyn Garden City, Herts., U.K.) by a method similar to that described by Hayes and Clarkson (1982). Assays were performed at 37°C. Samples were preincubated with GSH and the reactions were started by the addition of CDNB. The final substrate concentrations were 2mM and 1mM for GSH and CDNB respectively in a reaction volume of 0.25ml containing 0.1M-sodium phosphate buffer, pH 6.5. The reactions were monitored by initial measurement of the absorbance at 340nm, 10 seconds after

mixing, followed by 7 absorbance measurements at 5 second intervals. Reaction rates were determined by an integral kinetic data programme, which performed linear-regression analysis on the absorbance readings from each cuvette.

2.11.2 Glutathione peroxidase (GSHPx) assay

(a) Total glutathione peroxidase (tGSHPx) activity

Total GSHPx activity in the soluble fraction of cell samples was determined using cumene hydroperoxide as the substrate. The method used was that of Paglia and Valentine (1967) but adapted for use on a Cobas Fara centrifugal analyser. The reaction buffer contained 60mM Tris-HCl (pH 7.6), 0.12mM EDTA, 1mM sodium azide, 0.33mM NADPH, 1.3mM GSH and 1.3units/ml glutathione reductase (Sigma, Poole, Dorset). To this were added 10µl of cell lysate (cytosolic fraction). The inclusion of GSSG and glutathione reductase in the reaction mixture ensure that GSH is maintained at a constant concentration throughout the assay. After a 5 minute incubation the reaction was started by the addition of cumene hydroperoxide. Stock substrate solution consisted of 390µl cumene hydroperoxide in 18.75ml ethanol made up to 25ml with distilled water. The final concentration of substrate in the reaction mixture was 1.2mM. A water blank value was subtracted for each determination. The rate of the reaction was determined by following the decrease in absorbance at 340nm (i.e. oxidation of NADPH) at 37°C.

(b) Selenium-dependent (sGSHPx) activity

The assay for sGSHPx activity was essentially the same as for total tGSHPx activity, except that cumene hydroperoxide was replaced with 0.6mM hydrogen peroxide as substrate.

2.11.3 Glutathione reductase (GR) assay

Glutathione reductase activity was measured in the cytosolic fractions of cell samples using the fluorometric method of Weiss *et al* (1980) (equation 1).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+ \qquad (1)$$

The reaction mixture consisted of 67mM sodium phosphate buffer (pH 6.7), 0.5mM NADPH, 2.0mM nicotinamide, 3.0mM GSSG and 10µl of sample in a final volume of 40µl. The mixture was incubated at 37°C for 15 minutes and the reaction stopped

by the addition of 40µl of 0.1N HCl which also has the effect of destroying any unoxidised NADPH (Lowry and Passonneau, 1972). Fluorescence of NADP was induced by adding 1.0ml of 6N NaOH containing 0.03% H_2O_2 and heating to 60°C for 10 minutes. Fluorescence intensities were measured using a Perkin-Elmer spectrofluorometer (Model LS-3) at excitation 360nm and emission 460nm. A range of concentrations of NADP (12.5, 25, 50, 100 and 200µM) were used to generate a standard curve with which to calculate NADPH oxidation rates and, therefore, GR activity. A blank was included in the assay, consisting of reaction mixture with the sample omitted. The reading obtained from the blank was subtracted from that of the standards (prior to drawing the standard curve) and from those of the samples.

2.11.4 γ-glutamyl transpeptidase (γ-GT) assay

The fluorometric method described by Smith *et al* (1979) was used to measure γ-GT activity in the particulate fraction of cell lysates. Cell lysates were prepared as described in section 2.2. The particulate fraction was used for this assay. L-γ-glutamyl-7-amino-4-methyl coumarin (L-γ-glutamyl-AMC) (Universal Biologicals, Cambridge) was used as substrate. γ-GT catalyses removal of the γ-glutamyl moiety of this compound. This results in the formation of the product, 7-amino-4-methyl coumarin (AMC) (equation 2).

$$\text{L-}\gamma\text{-glutamyl-AMC} + \text{glycylglycine} \xrightleftharpoons{\gamma\text{-GT}} \text{L-}\gamma\text{-glutamyl-gly-gly} + \text{AMC} \qquad (2)$$

Samples were diluted with 0.1M ammendiol/HCl buffer (pH 8.6) (Ammendiol: 2-amino-2-methyl-1,3,propandiol, was obtained from Sigma, Poole, Dorset). Stock substrate solution was prepared by suspending L-γ-glutamyl-AMC in methoxyethanol (Aldrich Chemical Co., Gillingham) to give a concentration of 10mM. Homogeneity was obtained by brief sonication of the suspension. The stock solution was diluted with ammendiol/glycylglycine/triton buffer (which contained 100ml ammendiol buffer, 20mM glycylglycine and 100µl triton X-100) giving a final concentration of 0.2mM. Aliquots (100µl) of the samples to be tested were incubated in glass tubes at 37°C for 10 minutes with 250µl of 0.2mM L-γ-glutamyl-AMC. The reaction was stopped by the addition of 2.0ml of ice cold 0.05M glycine buffer (pH 10.4). Fluorescence was determined at room temperature using a Perkin-Elmer Model LS-3 spectrofluorometer at excitation 370nm and emission 440nm.

To generate a standard curve, the fluorescent product AMC (Sigma, Poole, Dorset) was diluted from a 10mM stock with ammendiol/glycylglycine/triton buffer.

A range of concentrations (2 to 20μM) was prepared and their fluorescence measured. Since L-γ-glutamyl-AMC is likely to be a substrate for non-specific peptide hydrolases the assay was verified by incubation of samples with the specific γ-GT inhibitor, 5mM serine/10mM borate at 37°C for 10 minutes (Tate and Meister, 1978). This was found to inhibit AMC formation completely.

2.11.5 γ-glutamyl cysteine synthetase (γ-GCS) assay

The method used to measure γ-GCS activity was based on that of Seelig and Meister (1984) and was adapted for use on a Cobas Fara centrifuge analyser. γ-GCS activity was determined from the rate of formation of ADP (assumed to be equal to the rate of oxidation of NADH) as calculated from the change in absorbance at 340nm (equations 3, 4, 5).

$$\text{L-glutamate + L-}\alpha\text{-aminobutyrate + ATP} \xrightarrow{\gamma\text{-GCS}} \gamma\text{-L-glutamyl-L-}\alpha\text{ -aminobutyrate + ADP + Pi} \quad (3)$$

$$\text{ADP + Pi + phosphoenolpyruvate + ATP} \xrightleftharpoons{\text{pyruvate kinase}} \text{pyruvate + ATP} \quad (4)$$

$$\text{pyruvate + NADH} \xrightleftharpoons{\text{lactate dehydrogenase}} \text{lactate + NAD} \quad (5)$$

The reaction mixture consisted of the following: 100mM Tris-HCl (pH 8.2), 2mM EDTA, 50mM KCl, 40mM $MgCl_2$, 10mM ATP, 10mM L-glutamate, 5mM L-γ-glutamyl-α-aminobutyrate. 10μl of cell lysate (cytosolic fraction) were added to the sample cuvettes. The reaction was allowed to proceed for 5 minutes before the addition of 0.2mM NADH (Sigma, Poole, Dorset), 17μg pyruvate kinase (Sigma Chemical Co.), 17μg lactate dehydrogenase (Sigma Chemical Co.) and 2mM phosphoenolpyruvate. The change in absorbance at 340nm gave the activity of γ-GCS.

2.11.6 Glutathione synthetase (GS) assay

Glutathione synthetase activity was determined at 37°C by measuring the rate of ADP formed during glutathione synthesis, by a method similar to that described by

Oppenheimer *et al* (1979). This assay was adapted for use on a Cobas Fara centrifugal analyser. Briefly, the primary reaction mixture (40µl) contained 100mM Tris-HCl (pH 8.2), 2mM EDTA, 50mM KCl, 20mM $MgCl_2$, 10mM ATP, 5mM glycine, 5mM γ-L-glutamyl-L-α-aminobutyrate. The reaction (equation 6) was initiated by the addition of 5µl of cell lysate (cytosolic fraction).

$$\gamma\text{-L-glutamyl-L-}\alpha\text{-aminobutyrate} + \text{glycine} + \text{ATP} \xrightleftharpoons{GS} \text{glutathione} + \text{ADP} + \text{Pi} \quad (6)$$

The reaction was allowed to proceed for 5 minutes before the addition of 0.52mM phosphoenol pyruvate, 0.2mM NADH and pyruvate kinase (1 unit), in 250mM potassium phosphate, 20mM $MgCl_2$ and 50mM KCl (pH 7.0). Following a 30 second incubation, the final reaction was initiated by the addition of 20µl of lactate dehydrogenase (25 units/ml in 250mM potassium phosphate, 40mM $MgCl_2$, 50mM KCl, pH 7.0). The formation of NAD was monitored by measuring the change in absorbance at 340nm as described for the assay for γ-GCS activity.

2.11.7 Quinone acceptor oxidoreductase (QAO) activity

QAO activity was measured using menadione as substrate and cytochrome c as the terminal electron acceptor. The enzyme assay mixture contained the following: 500µM NADH, 70µM cytochrome c and 10µM menadione. A 10µl aliquot of cell extract was added to 1ml of assay mixture. The initial rate of change in absorbance at 550nm was measured. A 10µl aliquot of 10mM dicoumarol (a potent inhibitor of QAO) was added to 10µl of cell extract. To this was added 1ml of reaction mix and the initial rate in change of absorbance measured. Specific QAO activity was given by the formula $[(a-b) \times 10^2]$ per mg/ml protein, where a = initial rate of change in absorbance with cell extract and b = initial rate of change in absorbance with cell extract and dicoumarol.

CHAPTER 3

CHARACTERISATION OF MENADIONE-RESISTANT CELL LINES

3.1 INTRODUCTION

In order to study the genetic changes that generate resistance to oxidants in eukaryotic systems, cell lines that are resistant to menadione have been isolated. The mechanisms of defence against oxidative stress have been thoroughly investigated in prokaryotes but there is less published work on the effects of oxidative stress in mammalian cells. Menadione was chosen as the selective agent for a number of reasons. Firstly, it has been used extensively as a model oxidant in studies involving prokaryotes. Secondly, the mechanisms of menadione-induced cytotoxicity have been investigated in a variety of model systems. The model most often used has been freshly isolated hepatocytes rather than cultured cells but the results are still relevant to the present study. Another reason for choosing menadione is that it is a quinone and this characteristic is one that it shares with a number of commonly used chemotherapy agents (see figure 3.1). The mechanisms of cellular defence against menadione might, therefore, shed some light on clinically important aspects of drug resistance.

The aims of this chapter are to summarise what is known about the toxicity of menadione, to describe the methods used to isolate menadione-resistant cells and to record their morphological characteristics, protein composition (as determined by two-dimensional electrophoresis) and cross-resistance patterns to both chemical oxidants and the physical agents, ionising radiation and heat.

3.1.1 Clinical studies of menadione

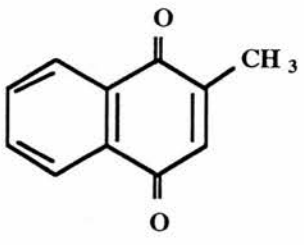
Menadione is a synthetic derivative of phylloquinone (vitamin K). It and a number of related naphthoquinones such as synkavit, 2-methyl-1,4-naphthoquinol-bis-disodium phosphate, have been investigated in the past as possible chemotherapeutic agents. The structures of menadione and synkavit are shown in Figure 3.1. The earliest reports of the cytotoxic effects of menadione and related compounds appeared in the 1940s and 1950s. Meier and Allgower (1945) found that

menadione inhibited the growth of chick fibroblast cultures and Bellairs (1954) reported that synkavit inhibited mitoses and so caused developmental abnormalities in chick embryos. However, menadione and its derivatives did not invariably act as mitotic inhibitors in all model systems tested. Gellhorn and Gagliano (1950), for example, found that intraperitoneally administered synkavit had no effect on the growth of murine tumours. Synkavit was used in many of these early studies because of its efficient uptake into cells. Once inside the cell, it is believed to interact with thiols to form menadiol, which on oxidation releases the active form, menadione.

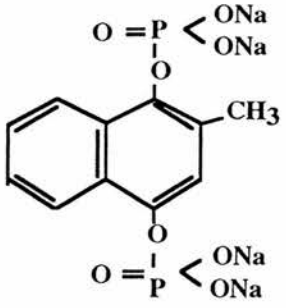
Mitchell and Simon-Reuss (1947) were the first to demonstrate that synkavit potentiated the effect of radiation on cultured cells. As a result of these studies synkavit was selected for investigation as a radiosensitiser in radiotherapy. Early clinical studies of the effects of menadione were carried out by Mitchell and co-workers at the Department of Radiotherapeutics, Cambridge, where the effect of synkavit in combination with radiotherapy in patients with inoperable bronchogenic cancer was tested (Mitchell *et al*, 1965). Another clinical trial investigating the effect of synkavit in combination with radiotherapy for advanced bronchogenic carcinoma was reported in 1962 by Deeley (Deeley, 1962). Eighteen patients were treated with radiotherapy alone and eighteen with radiotherapy together with synkavit which was injected prior to each fraction of treatment. At six months those who were given combined treatment had a 50% survival compared with 25% in the radiotherapy only arm of the trial. By one year the survival advantage of the combined treatment group had disappeared. This and other early trials of synkavit suggested a slight survival advantage for those patients given combination treatment (Krishnamurthi *et al*, 1967). However, these trials would not have met the rigorous requirements for a well-conducted study by current standards and at least one trial failed to show a radiosensitising effect of synkavit (Evans and Todd, 1969).

As well as being used as a sensitiser of external beam radiotherapy, synkavit was labelled with radioactive isotopes and then given intravenously. In one study, the I^{131}-labelled compound, 6-I^{131}-iodo-synkavit, was administered to fifty-one patients with advanced tumours (Marian *et al*, 1969). Selective uptake of the quinone by tumour was demonstrated in some cases of carcinoma of the colon, stomach, pancreas and in one kidney cancer. The distribution of radioactivity observed on radio-isotope scanning correlated, in general, with the known distribution of active tumour. A total of 179 patients with a variety of advanced, recurrent inoperable histologically verified malignant tumours and reticuloses were treated with tritiated derivatives of radio-labelled synkavit and a definite decrease in size of the tumour was observed in 14% of them (Mitchell, 1973).

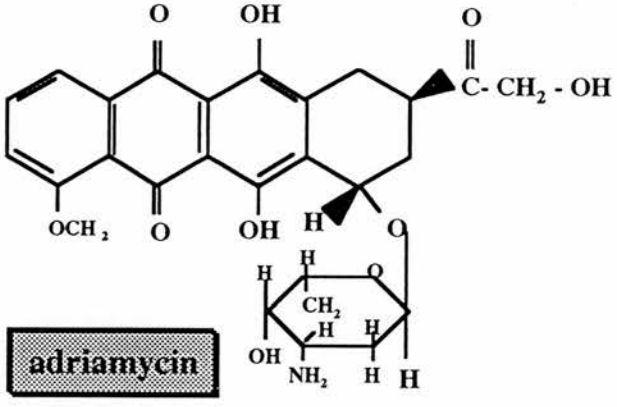
Figure 3.1 *Structure of menadione, synkavit and quinone cytotoxic compounds*



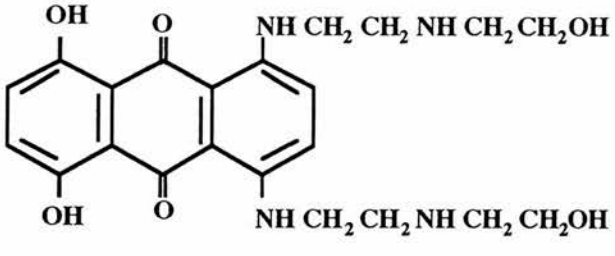
menadione



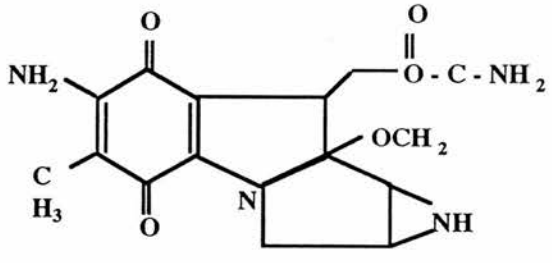
synkavit



adriamycin



mitoxantrone



mitomycin C

There has been renewed interest in menadione as a cytotoxic agent in the last decade. It has been shown to possess anticancer effects in rodent (Waxman and Bruckner, 1982) and human (Noto *et al*, 1989) cancer cell lines and in patient tumour explants (Chlebowski *et al*, 1985). Nutter *et al* (1991) reported a study of the cytotoxic effects of menadione on a panel of human tumour cell lines against which it showed a broad spectrum of antitumour activity. Menadione was found to be equally toxic towards a P-glycoprotein (Pgp) expressing leukaemia cell line (CEM-VBL) as towards the parental line from which it was derived. In other words, menadione did not appear to be a substrate for the Pgp drug efflux pump. This was despite the fact that the CEM-VBL cell line had been shown to be resistant to other chemotherapy drugs which possess a quinone structure, such as adriamycin and mitoxantrone. The inability of Pgp to interfere with menadione-induced cytotoxicity points to a possible role for menadione in the salvage treatment of cancer patients who have been heavily pretreated and whose tumours show resistance to several drugs. Nutter *et al* found that menadione was synergistic with 5FU, bleomycin, cisplatin and dacarbazine. Menadione has been tested in animals and its toxicity was found to be tolerable (Gold, 1986).

Menadione may have other clinical uses. It has, for example, been proposed as a treatment for disorders of the mitochondrial respiratory chain, the mitochondrial myopathies. In the commonest of these there is impairment of Complex one (ubiquinone reductase). Since it bypasses Complex one, menadione is able to compensate for defects at this level. Rat models of the mitochondrial myopathies have been generated by chronically treating rats with diphenylene iodonium. When given menadione these animals showed improved muscle function, weight gain and improved survival compared with untreated controls.

3.1.2 Menadione toxicity

The mechanisms of menadione-mediated toxicity have been extensively studied. It has been observed to cause a depletion of glutathione (Di Monte *et al*, 1984b), NADPH (Bellomo *et al*, 1982), and ATP (Redegeld *et al*, 1990), loss of protein sulphydryl groups (Bellomo *et al*, 1987), an increase in cytosolic free calcium (Nicotera *et al*, 1988), DNA damage (Nutter *et al*, 1992) and stimulation of the hexose monophosphate shunt (Smith *et al*, 1987). The relative contribution of each of these to the loss of cell viability is unclear.

(a) Relative importance of redox-cycling and sulphydryl arylation

In a number of studies an attempt has been made to assess the relative importance of the two main processes by which menadione mediates toxicity, redox-cycling and sulphydryl arylation. In one such study the effects of menadione and that of 2,3-dimethoxy-1,4-naphthoquinone (2,3-dioMe-1,4-NQ) were compared (Gant *et al*, 1988). The ability of the two compounds to redox-cycle was assessed by measuring NADPH oxidation. Their ability to arylate nucleophiles was measured by incubating them with glutathione and then measuring the amount of free GSH remaining after 30 minutes. 2,3-dioMe-1,4-NQ was found to redox-cycle as rapidly as menadione but unlike menadione it does not arylate nucleophiles. When added to freshly isolated rat hepatocytes, menadione at a concentration of 200μM and 2,3-dioMe-1,4-NQ at a concentration of 500μM both led to rapid depletion of glutathione. The fall in glutathione concentration was more profound with menadione than with 2,3-dioMe-1,4-NQ. In both cases, depletion of GSH was accompanied by equivalent GSSG formation suggesting that most of the GSH depletion resulted from redox-cycling rather than as a result of arylation reactions. Menadione was significantly more toxic than 2,3-dioMe-1,4-NQ. Menadione at a concentration of 200μM reduced the surviving cell fraction to 50% by one hour whereas the same degree of cell kill was only achieved with 200μM 2,3-dioMe-1,4-NQ after a three and a half hour incubation.

In a similar study, Toxepeus *et al* (1993) also addressed the question of the relative importance of redox-cycling and sulphydryl arylation. Freshly isolated rat hepatocytes were again used to study the effects of menadione and 2,3-dioMe-1,4-NQ. In addition, 1,4-naphthoquinone (NQ) was studied. This agent has a greater arylation capacity than menadione and 2,3-dioMe-1,4-NQ but redox-cycles at the same rate as menadione. A comparison of the ability of these three compounds to induce loss of cell viability showed that NQ was more potent than menadione and menadione more potent than 2,3-dioMe-1,4-NQ. The arylation capacity of a particular quinone therefore appears to determine its cytotoxic potential. Protein thiol loss was extensive following treatment with NQ, moderate following treatment with menadione and did not occur with 2,3-dioMe-1,4-NQ. This again reflects the arylating capacity of the three quinones. The conclusion that can be drawn from these studies is that the toxicity induced by menadione is partly due to its electrophilic nature and thus its capacity to arylate. Redox-cycling and the induction of oxidative stress is only one aspect of menadione-induced cell damage.

(b) Menadione-induced DNA damage

The induction of single-strand DNA breaks have been demonstrated in rat hepatocytes and human fibroblasts treated with menadione (Morrison *et al*, 1984; Morrison *et al*, 1985b). Nutter *et al* (1992) used alkaline and neutral elution methods, to demonstrate that menadione induced both single-strand and double-strand DNA breaks in human (MCF-7) cells. There was good correlation between the numbers of both types of DNA break and cytotoxicity. The addition of catalase to the growth medium reduced both single-strand and double-strand through, it was assumed, the scavenging of extracellular H_2O_2. Catalase also attenuated menadione-induced cell death, suggesting that H_2O_2 and $OH\cdot$ play important roles in menadione toxicity.

(c) Energy metabolism

Cells contain two main pools of glutathione, one in the cytosol and one in the mitochondria. Mitochondrial glutathione accounts for only 10-15% of the total but is considered to be crucial for the maintenance of cell viability. The depletion of mitochondrial glutathione associated with menadione toxicity, leads to a loss of mitochondrial function and so to the cessation of ATP production. Other mechanisms by which menadione may decrease ATP-generation include the inhibition of glycolytic enzymes by sulphydryl arylation and stimulation of the hexose monophosphate shunt which in its turn reduces the amount of glucose-6-phosphate available for glycolytic ATP generation. ATP-depletion leads to a profound loss of cellular homeostatic control. For example, it results in a disturbance of homeostatic control by the inhibition of Na^+, K^+-ATPases and Ca^{2+}-ATPase. Furthermore the maintenance of ATP levels is important for the polymerisation of microfilaments and microtubules so that a deficiency of ATP leads to the disruption of the cytoskeleton. The depletion of ATP induced by menadione may therefore contribute to the cytoskeletal changes that are a frequently reported sequelae of this compound.

Redegeld *et al* (1992) set out to examine the relative importance of ATP depletion and of glutathione and NADPH depletion in menadione-induced cytotoxicity. Freshly isolated hepatocytes from fasted and fed rats were used. It was found that whatever the nutritional state of the animals from which the hepatocytes were obtained, GSH and NADPH fell rapidly following treatment with menadione. Except that the resting level of GSH was higher in fed than in fasted animals there was no difference in the kinetics of glutathione metabolism between the two. The

intracellular concentration of NADPH fell instantaneously following the addition of 150μM menadione and this was equally true of both fed and fasted animals. The ATP content of the hepatocytes fell to 50% of the starting value by 18 minutes in fasted and by 65 minutes in fed animals and there was a concomitant increase in the concentrations of ADP and AMP. The decline in ATP concentrations always preceded loss of cell viability and correlated well with the time and extent of cell killing (unlike the depletion of NADPH and GSH). When hepatocytes were incubated with the substrates for mitochondrial ATP generation (pyruvate, oxaloacetate and glutamine) menadione-induced ATP depletion was postponed and menadione cytotoxicity attenuated (Redegeld *et al*, 1990). Loss of cell viability (as assessed by the LD_{50}) was delayed from 77 minutes in the absence of the substrates to 125 minutes in their presence. The observation of a strong correlation between ATP-depletion and menadione-induced cytotoxicity suggests that disturbance of ATP is an important aspect of the metabolism of this quinone.

(d) Cytoskeletal changes

Dramatic changes in cell surface morphology consisting of the appearance of protrusions of the plasma membrane may occur as part of a general cellular response to stress. Such alterations in cell surface morphology have been observed following X-irradiation (Borek and Fenoglio, 1976), anoxia (Lemasters *et al*, 1987) and hyperthermia (Coss *et al*, 1979). Bleb formation has often been detected after oxidative injury including that induced by quinone compounds (Jewell *et al*, 1982; Lemasters *et al*, 1987). As noted above, some quinones, and this includes menadione, deplete protein thiols through arylation. Some cytoskeletal protein SH groups are critically important for the structural integrity of the cell and it is therefore not surprising that alterations in the cytoskeleton and the plasma membrane are a frequently reported feature of quinone toxicity. Mirabelli *et al* (1988) showed that incubation of freshly isolated rat hepatocytes or of cultured cells with menadione was followed by the appearance of cell surface protrusions. The appearance of these morphological changes was preceded by biochemical alterations (particularly GSH depletion) and followed by cell death. To investigate further the changes underlying these structural changes, the cytoskeletal fraction was extracted from untreated and menadione-treated cells. Cytoskeletal protein composition was then analysed by SDS-PAGE electrophoresis. Two major differences between menadione-treated and control cells were noted. A protein that migrated like actin disappeared and a high molecular weight aggregate, which did not enter the gel, appeared in menadione-

treated cells. Pretreatment with the reductant β-mercaptoethanol prevented both the disappearance of the band corresponding to actin and prevented the appearance of the high molecular weight aggregate. This suggested that the latter contained actin molecules cross-linked by disulphide bonds. These data suggest that menadione is able to disrupt actin through arylation of SH groups and account for the loss of actin in the cytoskeletal fraction of menadione-treated cells and so for the cell surface changes that ensue.

Menadione-induced cell surface protrusions have been observed mainly in round and mitotic cells. A wide variety of cultured cells exhibited blebbing on treatment with menadione but the proportion of cells showing this feature varies greatly. In CG5 (a human breast carcinoma cell line) 42% of cells were blebbed and in K562 (a human erythroleukaemia cell line) 90% were blebbed (Malorni *et al*, 1991). These blebs are free of organelles and contain only a few ribosomes and amorphous material. Immunocytochemistry showed that the expression of some surface marker proteins (collagen IV, sialo-protein, β2-microglobulin, fibronectin) was reduced over the bleb surface while actin and keratin filaments appeared to be completely absent. These microfilament changes result in dissociation of the cytoskeleton from its anchoring domains in the plasma membrane and this generates sites of structural weakness where blebs can form. The microfilament system therefore seems to represent another important target for menadione cytotoxicity.

Menadione has been shown to cause disruption of cellular calcium homeostasis (Di Monte *et al*, 1984a). To investigate the relative contribution of altered intracellular calcium homeostasis in menadione-induced bleb formation, hepatocytes were treated with or without the intracellular calcium chelator, Quin-2 and then with 200μM menadione (Malorni *et al*, 1991). All cells developed blebs but in Quin-2 pretreated cells the onset of blebbing was delayed. This indicated that the increase in cytosolic free calcium was involved in the early stages of bleb formation. Complete protection from blebbing was obtained with the thiol reductant DTT indicating that alteration in cellular redox state brought about by menadione influences the cytoskeleton.

(e) Mode of menadione-induced cell death

Apoptosis is a distinct form of cell death involved in many physiological and pathological processes. Many agents that induce apoptosis are oxidants or are able to stimulate cellular oxidative metabolism (Buttke and Sandstrom, 1994). Conversely, many agents that inhibit apoptosis have antioxidant activity and enhance the

antioxidant defences. Oxidative stress may therefore precipitate apoptosis. Examples of physical agents that produce oxidative stress and apoptosis are ionising radiation and UV radiation. Low doses of hydrogen peroxide (10-100μM) induce apoptosis in a variety of cell types but high doses produce necrosis (Lennon *et al*, 1991). This lends weight to the suggestion of Duvall and Wyllie (1986) that the severity of the insult determines the form of cell death, apoptosis or necrosis. Menadione was observed to induce dose-related apoptotic cell death in two cell lines (FL5.12, an interleukin-3-dependent murine cell line and 2B4, a T-cell hybridoma) in a study by Hockenbery *et al* (1993).

3.2 STRATEGIES

3.2.1 Choice of parental cell lines

The two parental cell lines used for the isolation of menadione-resistant mutants were CHO-K1, derived from Chinese hamster ovary and EJ-WT, derived from human transitional cell carcinoma. A Chinese hamster ovary cell line was chosen because it is not fastidious in terms of culture requirements and a high plating efficiency is routinely achievable. The particular Chinese hamster ovary line used, CHO-K1, has been thoroughly characterised (Lewis *et al*, 1988b; Robson *et al*, 1986). We also wished to study a human malignant cell line as there was a relative paucity of published work on the effects of menadione in human cancer cells.

3.2.2 Strategies for isolation of menadione-resistant cell lines

(a) Pulse treatments of menadione

It has been suggested that the use of repeated, short exposures to drug is the preferable means of obtaining drug-resistant cell lines since this accurately reflects the clinical situation, in which patients are given cyclical treatment (Yang and Trujillo, 1990). Indeed this was the strategy used by Ngo and Nutter (1994) who have reported the characterisation of a menadione-resistant cell line derived from the human breast adenocarcinoma cell line, MCF-7.

The cell lines CHO-MRs40, CHO-MRs60, CHO-MRs80, CHO-MRs100, EJ-MRs40, EJ-MRs60, EJ-MRs80 and EJ-MRs100 were isolated by pulsed exposures to menadione. Preliminary cytotoxicity assays indicated that only a modest degree of resistance was achieved and that menadione-resistant cell lines obtained using this method reverted rapidly to wild-type phenotype between treatments. This is in

contrast to the work of Ngo who successfully obtained menadione-resistant cell lines using the pulse drug treatment method. There are, however, a number of important differences between the work of Ngo and the present study. Firstly a different parental cell line was used (the human breast adenocarcinoma cell line, MCF-7) and also the cells were exposed to shorter (one hour) pulses of menadione. Ngo used a total of ten pulse treatments over a twelve month period and found that further pulsing did not lead to a further increase in resistance.

(b) Continuous exposure to drug

The menadione-resistant cells CHO-MRc20, CHO-MRc30, CHO-MRc40, EJ-MRc20, EJ-MRc30 and EJ-MRc40 were isolated by continuous exposure of cells to menadione. The isolation of resistant cells by continuous exposure and subculturing in the presence of the drug has the disadvantage that it may be difficult to interpret the number of genetic changes associated with the final phenotype. However, this was the most successful of the methods used.

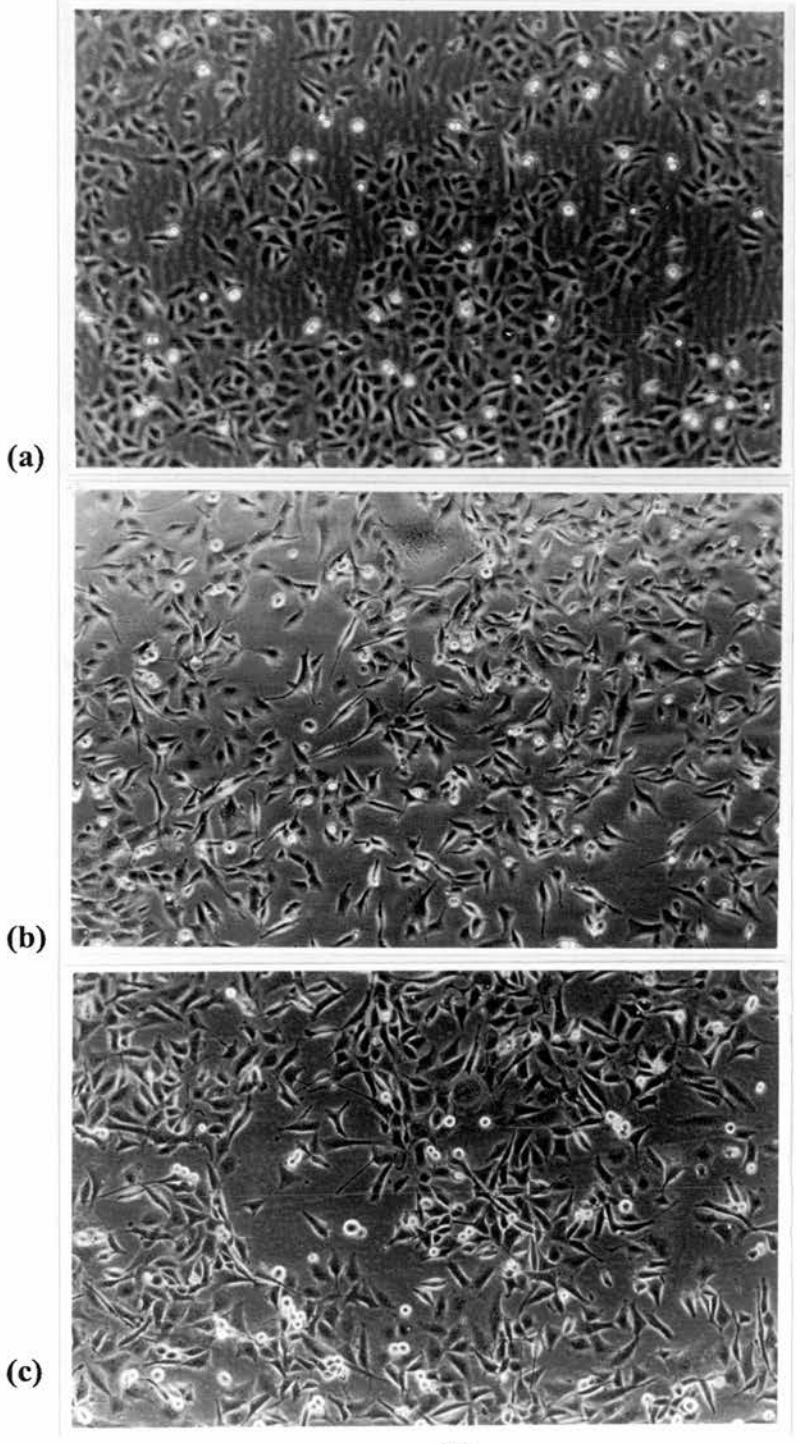
(c) Pretreatment with mutagen

The disadvantage of the use of a mutagenising agent is that it may inflict DNA damage at many sites and so affect several properties of the cell other than the mechanisms involved in the resistance phenotype itself. However in some instances a mutagen is necessary to elevate mutation frequency to a detectable level. EMS, the most commonly used mutagen, was used in this study as described in the section 2.1.2. However, the cell lines obtained in this way did not show a greater degree of resistance than those obtained by continuous exposure to the selective agent and so were not studied further.

3.3 RESULTS

3.3.1 Light microscopic appearance of menadione-resistant cells

Figure 3.2 Morphological appearance of (a) EJ-WT (b) EJ-MRc20 and (c) EJ-MRc30 cell lines. The menadione-resistant cells are larger and exhibit more cytoplasmic protrusions compared with the parental cell line. The cytoplasm of menadione-resistant cells is more granular in appearance than that of the wild type.



Figures 3.3 to 3.6 show a series of photographs of CHO cells in culture. As videomicroscopy was used to take these photographs the same microscopic field is shown in each picture. This allows changes in individual cells to be observed over time and following different treatments. It is of interest that no evidence of blebbing is seen either in CHO-K1 or CHO-MRc40 cells despite treatment with menadione. The mode of cell death in both cell lines appears to be necrosis.

The video films were analysed to give mean intermitotic times for the two cell lines. This is done by following individual cells on the film and since the time interval between each frame is known it is possible to calculate the intermitotic time. The results of these measurements are summarised in Table 3.1. In unstressed conditions, the resistant cells grow slightly more slowly than CHO-K1. The addition of menadione to the growth medium results in a dramatic slowing of cell growth and prolongation of the interval between mitoses. CHO-K1 cells undergo such rapid rounding up, detachment and then death after addition of menadione that it was not possible to measure the inter-mitotic time of this cell line in the presence of drug.

Table 3.1 *Intermitotic times of CHO-K1 and CHO-MRc40 cells*

Cell line	**Mean (SD) Intermitotic time (hours)**		**Range (hours)**
CHO-K1	13.6 (1.7)	n=25*	7.7 - 16.7
CHO-MRc40	14.4 (1.0)	n=13*	13 - 17.4
CHO-MRc40 (following menadione 25μM)	20.1 (4.8)	n=49*	14.6 - 35.8

* n = number of intermitotic times measured

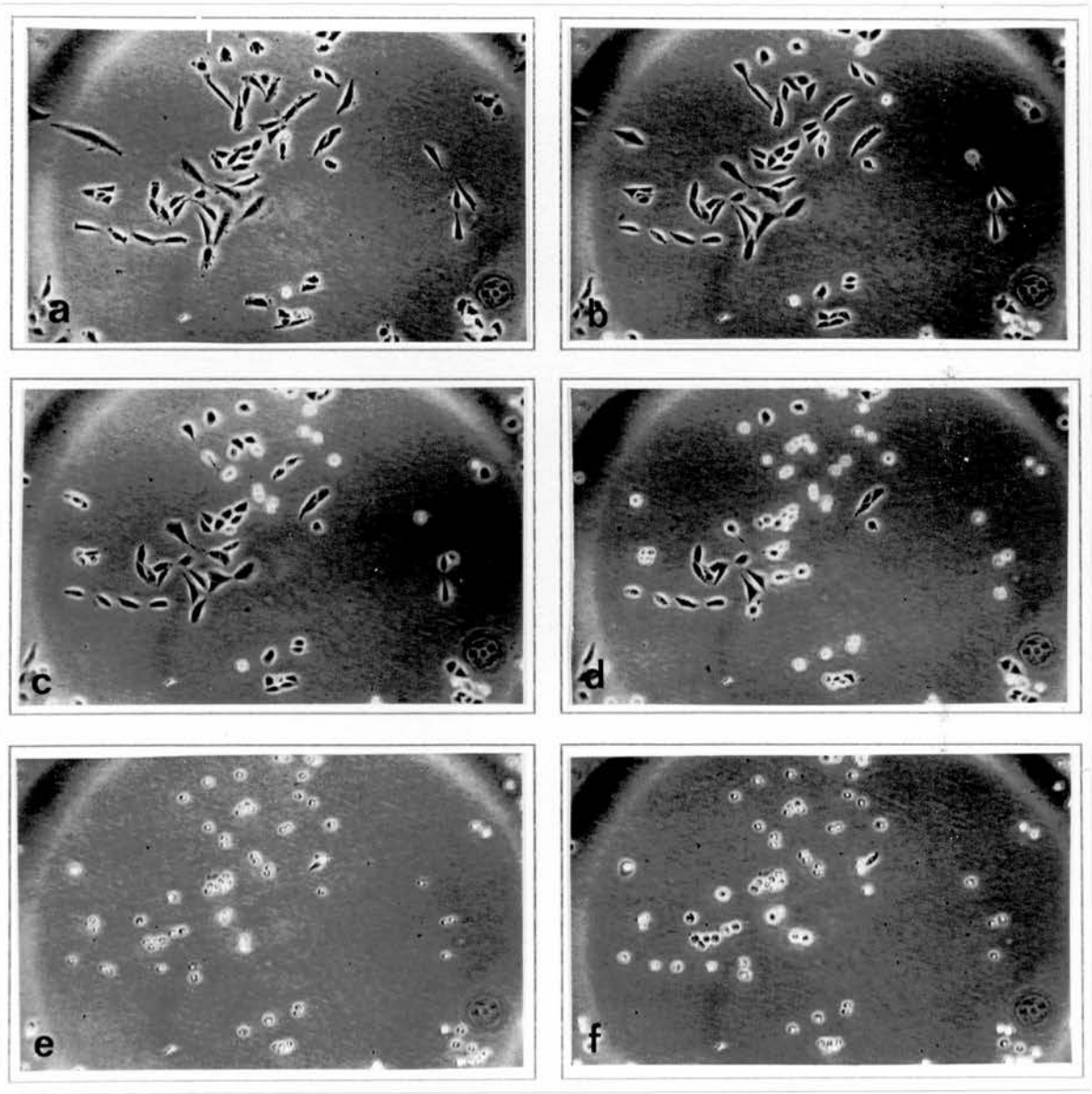


Figure 3.3 Photographs taken from a videomicroscopy recording of CHO-K1 cells growing in drug-free medium and then in the presence of 25μM menadione. Appearance of CHO-K1 cells (a) immediately before addition of menadione and (b) 2 hours (c) 3 hours (d) 4 hours (e) 15 hours and (f) 36 hours after the addition of menadione. Cells round up and detach soon after the addition of the drug and there is no recovery during the following 36 hours.

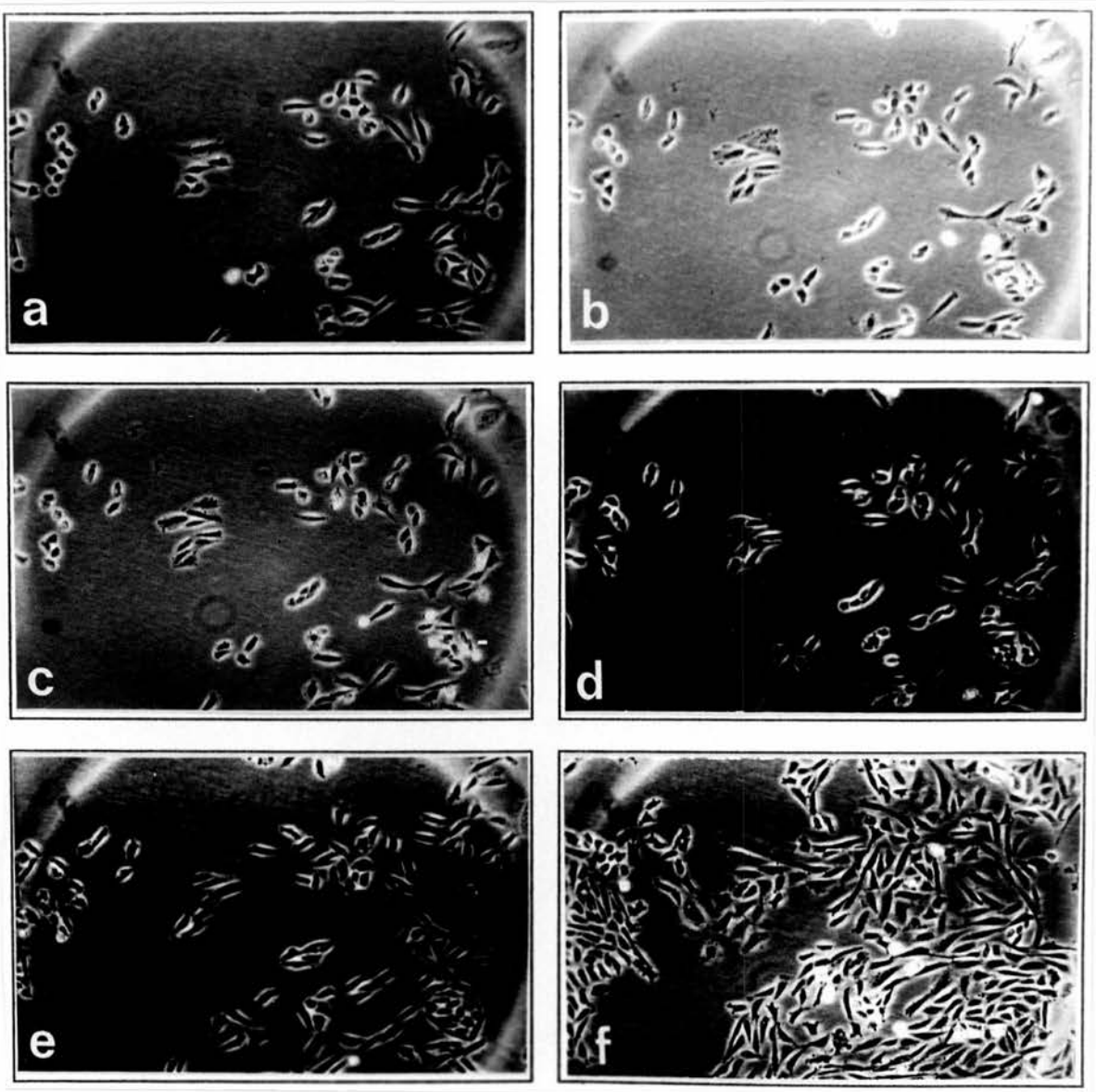


Figure 3.4 Photographs taken from a videomicroscopy recording of CHO-K1 cells growing in drug-free medium and in the presence of 25μM menadione. Cells were treated for 2 hours before the addition of menadione with 100mM *N*-acetyl cysteine (NAC). NAC is readily taken up by cells and by deacetylation forms cysteine and stimulates glutathione synthesis. Appearance of CHO-K1 cells (a) immediately before addition of menadione and (b) 2 hours (c) 3 hours (d) 4 hours (e) 15 hours and (f) 36 hours after the addition of menadione. These pictures are in sharp contrast to those of figure 3.3. The cells continue to grow and divide in the presence of menadione due to the protective effect of NAC.

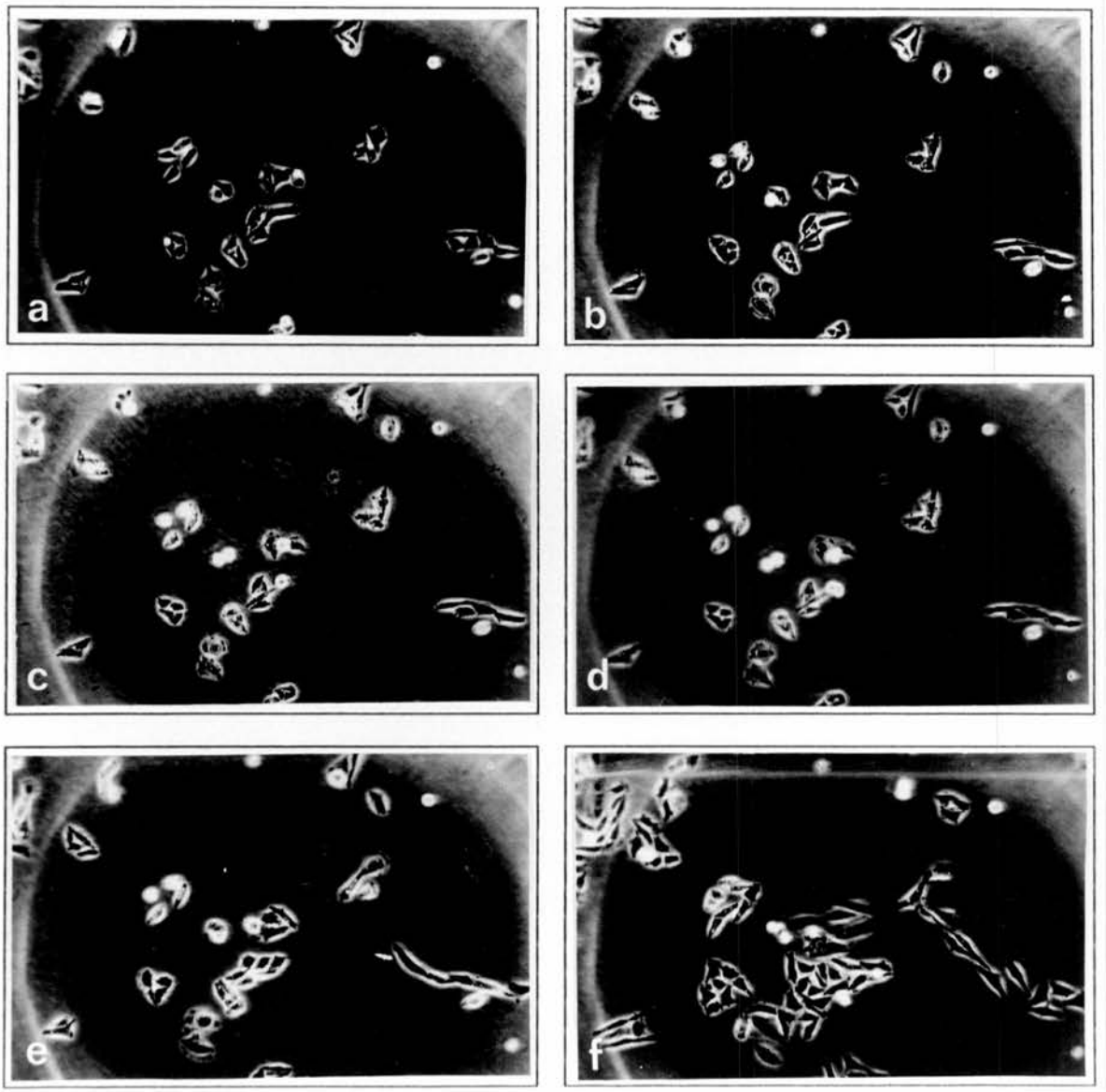


Figure 3.5 Photographs taken from a videomicroscopy recording of CHO-MRc40 cells growing in drug-free medium and in the presence of 25μM menadione. Appearance of CHO-MRc40 cells (a) immediately before addition of menadione and (b) 2 hours (c) 3 hours (d) 4 hours (e) 15 hours and (f) 36 hours after the addition of menadione. Unlike the CHO-K1 cells shown in figure 3.3, the menadione-resistant cells continue to grow and divide in the presence of drug although their mean intermitotic time lengthened from 14.4 hours (SD 1.0) to 20.1 hours (SD 4.8) following addition of menadione.

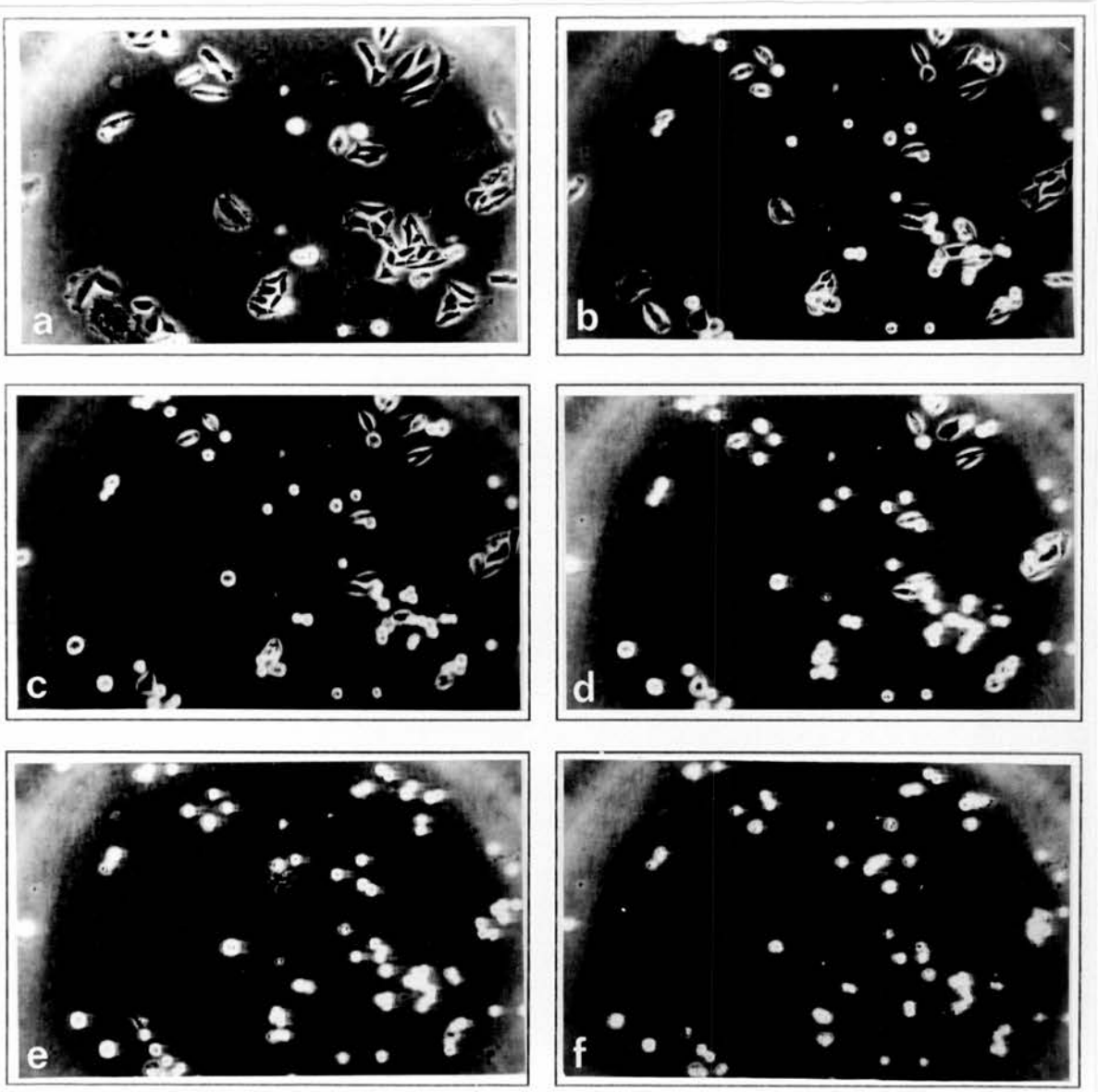


Figure 3.6 Photographs taken from a videomicroscopy recording of CHO-MRc40 cells that had been pretreated with buthionine sulphoximine (100μM for 24 hours) growing in menadione-free medium and then in the presence of 25μM menadione. Appearance of cells (a) immediately before addition of menadione and (b) 2 hours (c) 3 hours (d) 4 hours (e) 15 hours and (f) 36 hours after the addition of menadione. The response of glutathione-depleted CHO-MRc40 cells to menadione was similar to that of CHO-K1 cells. They detached and died very soon after the addition of drug.

3.3.2 Electron microscopic appearance of cells

Nucleated cells may show one or other of two stereotyped patterns when they die, apoptosis or necrosis. Apoptosis is the name that has been given to the process of physiological cell death (Duvall and Wyllie, 1986; Fesus *et al*, 1991) since it plays a role in processes such as embryonic morphogenesis and hormone-induced tissue remodelling. It is characterised by rounding up of the cell and disruption of junctions with adjacent cells. Fragmentation of the nucleus and cell shrinkage due to loss of cytoplasmic volume both take place. Internucleosomal cleavage of the chromatin results in the formation of DNA fragments with lengths that are integer multiples of 180 to 200 base pairs. These fragments appear as a distinctive "ladder" on electrophoretic DNA gels. Necrosis may be precipitated by a large number of stimuli including complement attack, viral infection, heat and bacterial toxins. The morphological characteristics of necrosis include swelling of the cell and condensation of the mitochondria as their inner membrane shrinks away from their outer membrane. Nuclear chromatin flocculates, the endoplasmic reticulum dilates and membrane blebbing is seen. Protein synthesis declines. Necrosis but not apoptosis stimulates an inflammatory response.

Severe oxidative stress results in the oxidation of both reduced glutathione and NAD(P)H, protein thiol modification, energy depletion and perturbation of intracellular ion homeostasis. Severe oxidative stress also gives rise to DNA damage and the stimulation of poly (ADP-ribose) polymerase activity. This may lead to the depletion of NAD^+ and ATP. The resulting cell death is of the necrotic type. Moderate oxidative stress may selectively affect enzymes involved in signal transduction. This may inappropriately activate a cell deletion programme (apoptosis).

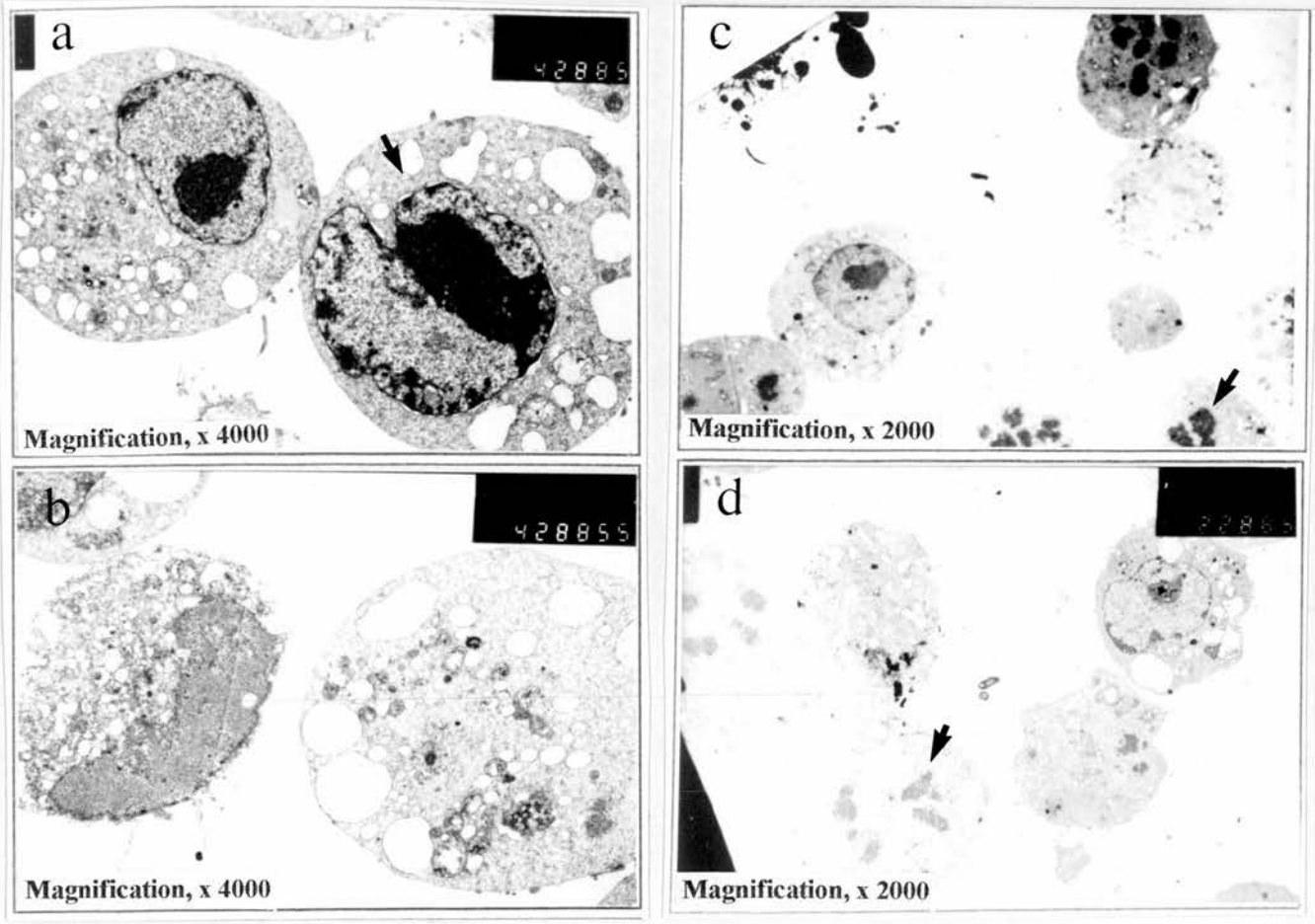
Figures 3.7 and 3.8 show the electron microscopic appearance of the menadione-resistant and -sensitive cell lines following treatment with menadione. CHO-K1 and CHO-MRc40 cells were treated with 40μM menadione for 6 hours. Most of the CHO-K1 cells shown in figure 3.7 (a) and (b) exhibit the features of necrosis. These are swelling of the cells, marked vacuolation of the cytoplasm and a disorganised appearance of the nucleus. In the cell marked with an arrow there is some evidence of nuclear fragmentation which is a feature of apoptosis. So in this particular cell there are features of both modes of death. By contrast the CHO-MRc40 cells shown in figure 3.7 (c) and (d) look normal and indeed several show chromosomal condensation indicating that they are undergoing mitoses (black arrows).

EJ-WT and EJ-MRc30 cells were treated with 30μM menadione (a lower

concentration was used for EJ-WT cells as they are less tolerant of the drug). EJ-WT cells in figure 3.8 (a) and (b) are necrotic and non-viable with marked disruption of the nuclei and vacuolation of the cytoplasm. EJ-MRc30 cells shown in figure 3.8 (c), although showing some signs of necrosis, appear to be viable.

Thus it appears that the mode of death of CHO-K1 and EJ-WT following treatment with menadione is necrosis. It seems that the mode of death induced by oxidative stress depends on the severity of the applied insult. In this case it would seem that the parental cell lines are experiencing extreme oxidative stress since necrosis is obvious. The appearance of a few of the features of apoptosis in one of the CHO-K1 cells suggests that had a slightly lower concentration of menadione been used then apoptosis may have been the predominant mode of death.

Figure 3.7 Electron microscopic appearance of a) and b) CHO-K1 and c) and d) CHO-MRc40 following treatment with 40μM menadione for 6 hours.



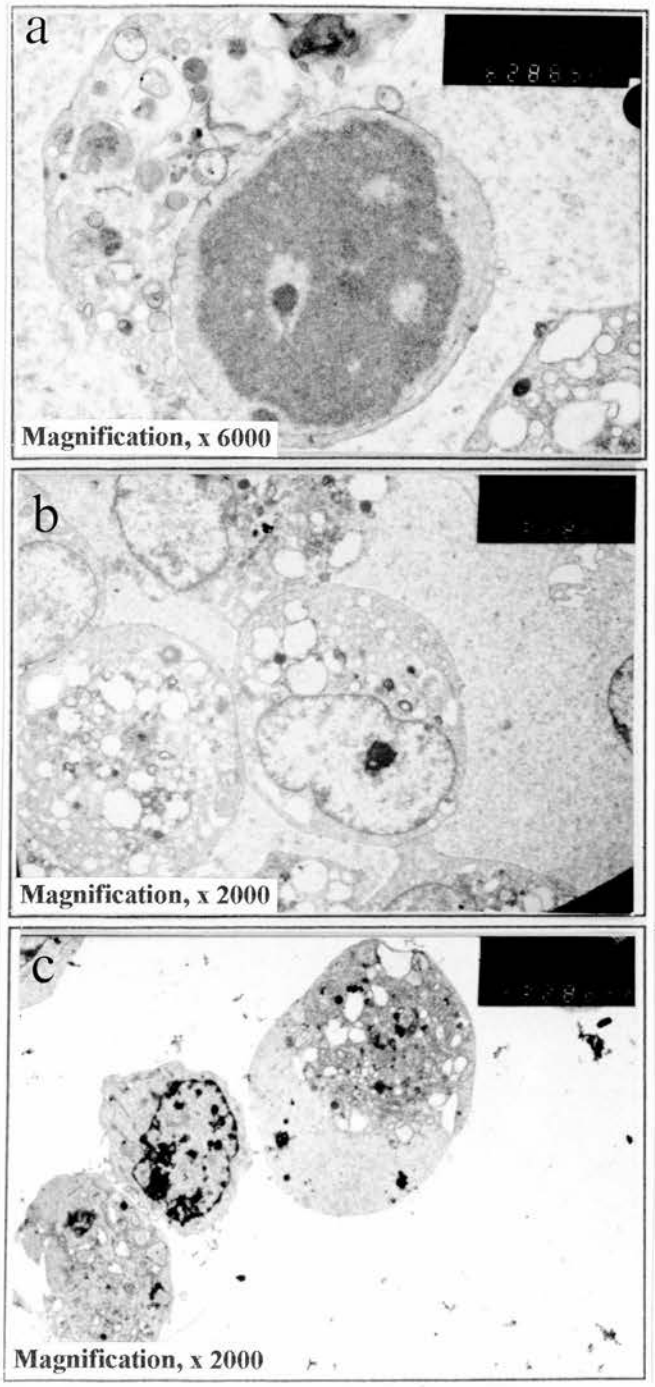


Figure 3.8 Electron microscopic appearance of a) and b) EJ-WT and c) EJ-MRc30 following treatment with 30μM menadione for 6 hours.

3.3.3 Resistance to menadione and other cytotoxic compounds

The cell survival curves for the EJ-MRc cell lines is shown in Figure 3.9 and those for the EJ-MRs cell lines are shown in Figure 3.10. It can be seen that the degree of resistance was greater in the cell lines that were derived by continuous exposure of cells to menadione compared with those that were treated by pulsed exposures to the drug. Figure 3.11(a) shows the dose-response curve for the most resistant of the CHO cell lines (CHO-MRc40) to menadione. This cell line was 1.8 fold resistant to menadione at the 50% survival level compared with the parental line. Also shown in Figure 3.11 are the survival curves for this cell line following treatment with agents to which it exhibited cross-resistance, namely menadione bisulphite, the sulphydryl-reactive compound, sodium arsenite and the oxidising agent, hydrogen peroxide. The LD_{50} values for these agents were 1.6, 1.6 and 1.9-fold higher than in control cells respectively. A slight increase in resistance to ethacrynic acid (1.3-fold), chlorambucil (1.3-fold) and to adriamycin (1.3-fold) was observed but there was no difference between the wild-type and drug-resistant cell lines in their sensitivity to paraquat, cadmium chloride or bleomycin (Figure 3.12). The LD_{50} is equal to the concentration of drug causing 50% cell death. LD_{50} values were taken directly from the graphs shown in Figures 3.11 and 3.12. These data are summarised in Table 3.2.

Survival curves were also obtained using the clonogenic assay for those agents to which CHO-MRc40 showed cross-resistance (Figure 3.13). In this assay cells were exposed to the drug for a shorter time (four hours) compared to the three day exposure used in the MTT assay. Using the clonogenic assay, the LD_{50} values for CHO-MRc40 treated with menadione, hydrogen peroxide and sodium arsenite were 7.8, 1.8 and 1.9-fold higher respectively than for CHO-K1.

3.3.4 Cross-resistance to heat

The effect of heating to 42°C on the survival of CHO-MRc and EJ-MRc cell lines compared to that of their respective parental cell lines is shown in Figures 3.14 and 3.15. The method used to obtain these data is described in section 2.1.5.

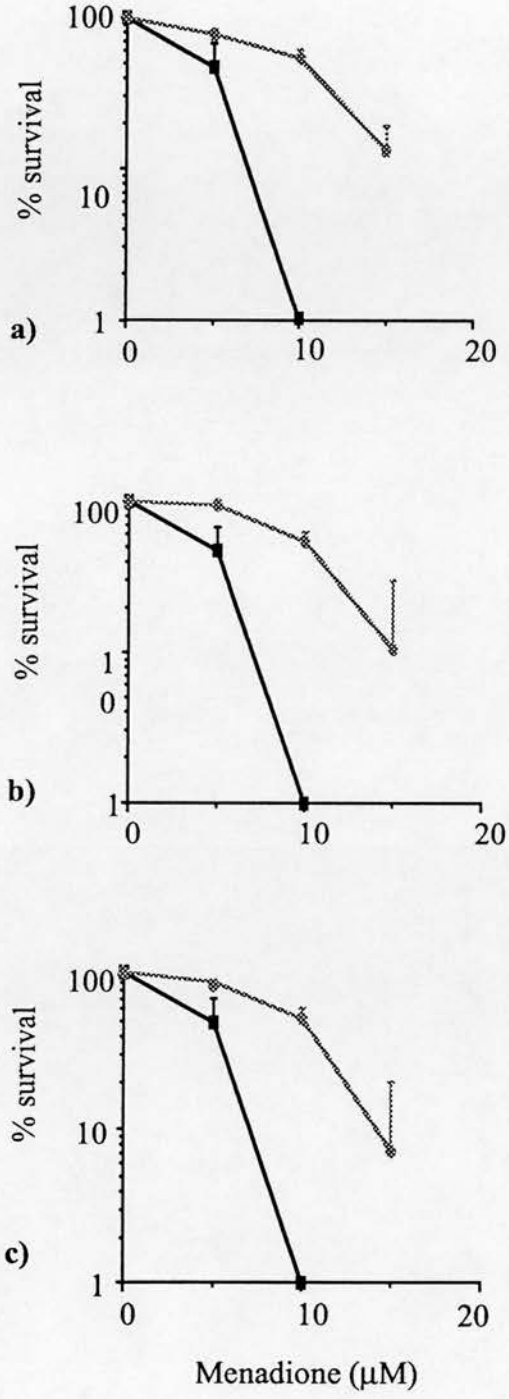


Figure 3.9 Cell survival curves based on MTT assays.
Resistance of EJ WT (■) and menadione-resistant cell lines (❋) to menadione **a)** EJ-MRc20 **b)** EJ-MRc30 and **c)** EJ-MRc40. All data points represent the mean and standard deviation of at least triplicate samples. Where error bars are not shown they come within the area of the plot symbol.

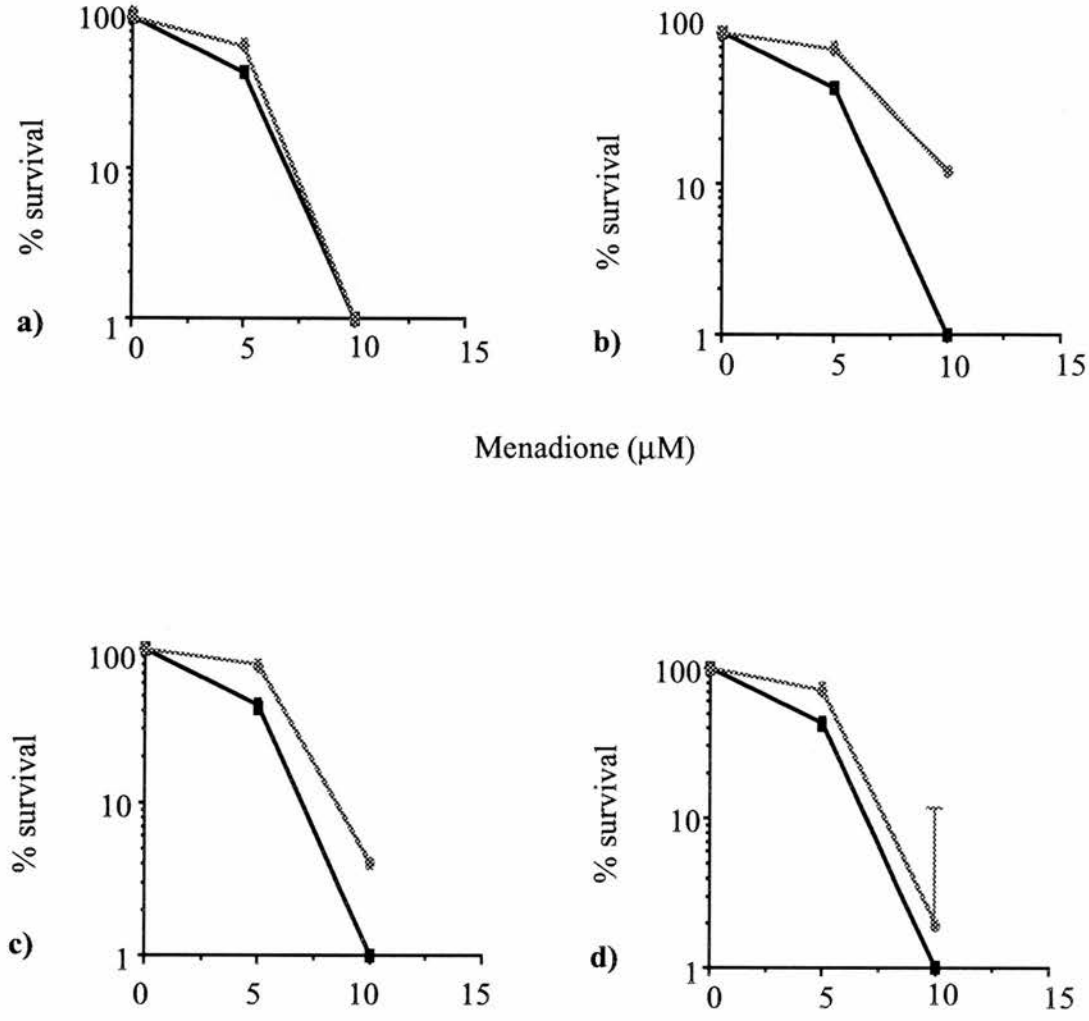


Figure 3.10 Cell survival curves based on MTT assays.
Resistance of EJ WT (■) and menadione-resistant cell lines (❋) to menadione **a)** EJ-MRs40 **b)** EJ-MRs60 **c)** EJ-MRs80 and **d)** EJ-MRs100. All data points represent the mean and standard deviation of at least triplicate samples. Where error bars are not shown they come within the area of the plot symbol.

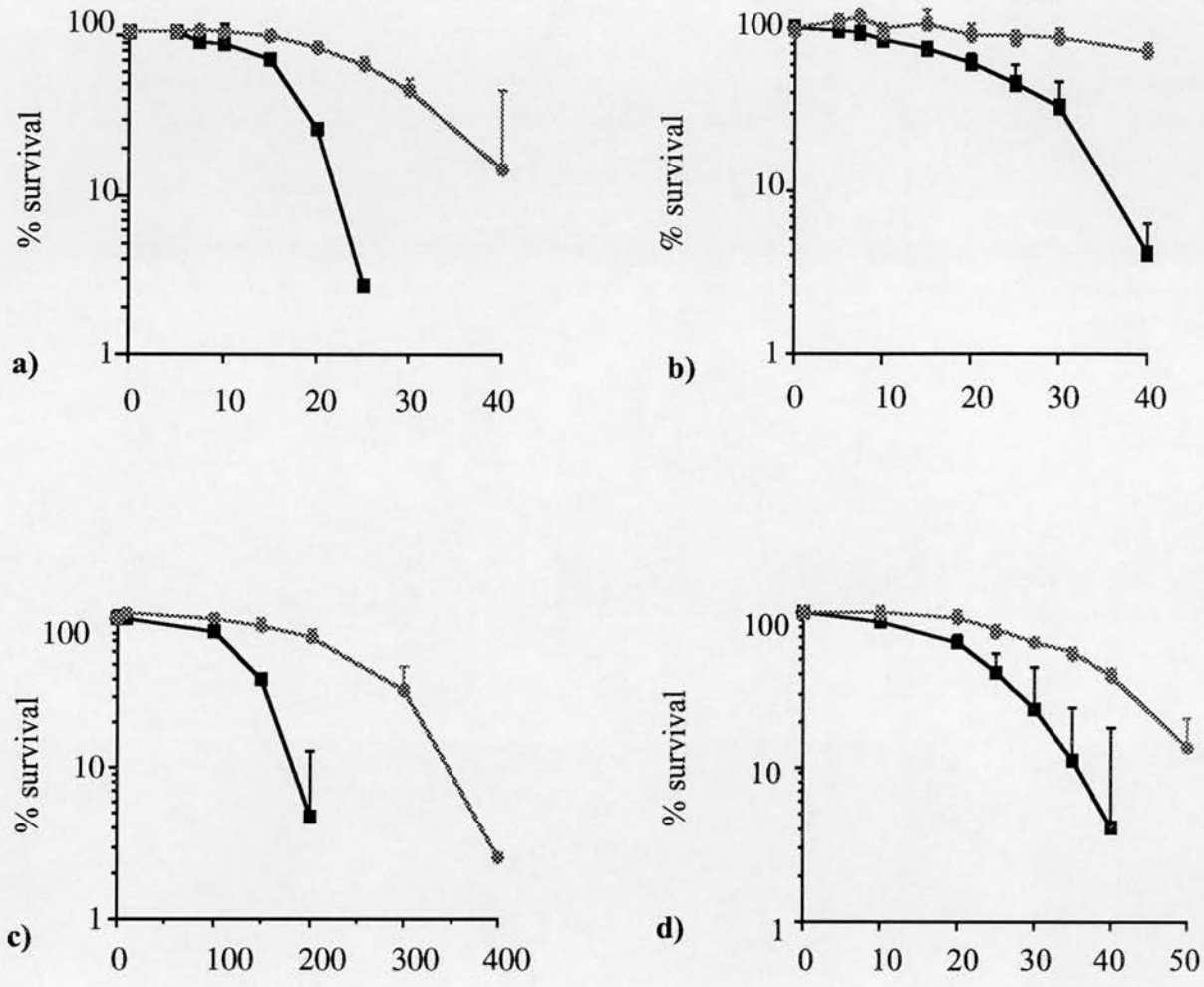


Figure 3.11 Cell survival curves based on MTT assays.

Sensitivity to the following agents was tested a) menadione b) menadione bisulphite c) hydrogen peroxide and d) sodium arsenite. CHO-K1 (■), CHO-MRc40 (❋). All drug concentrations are μM. All data points represent the mean and standard deviation of at least triplicate samples. Where error bars are not shown they come within the area of the plot symbol.

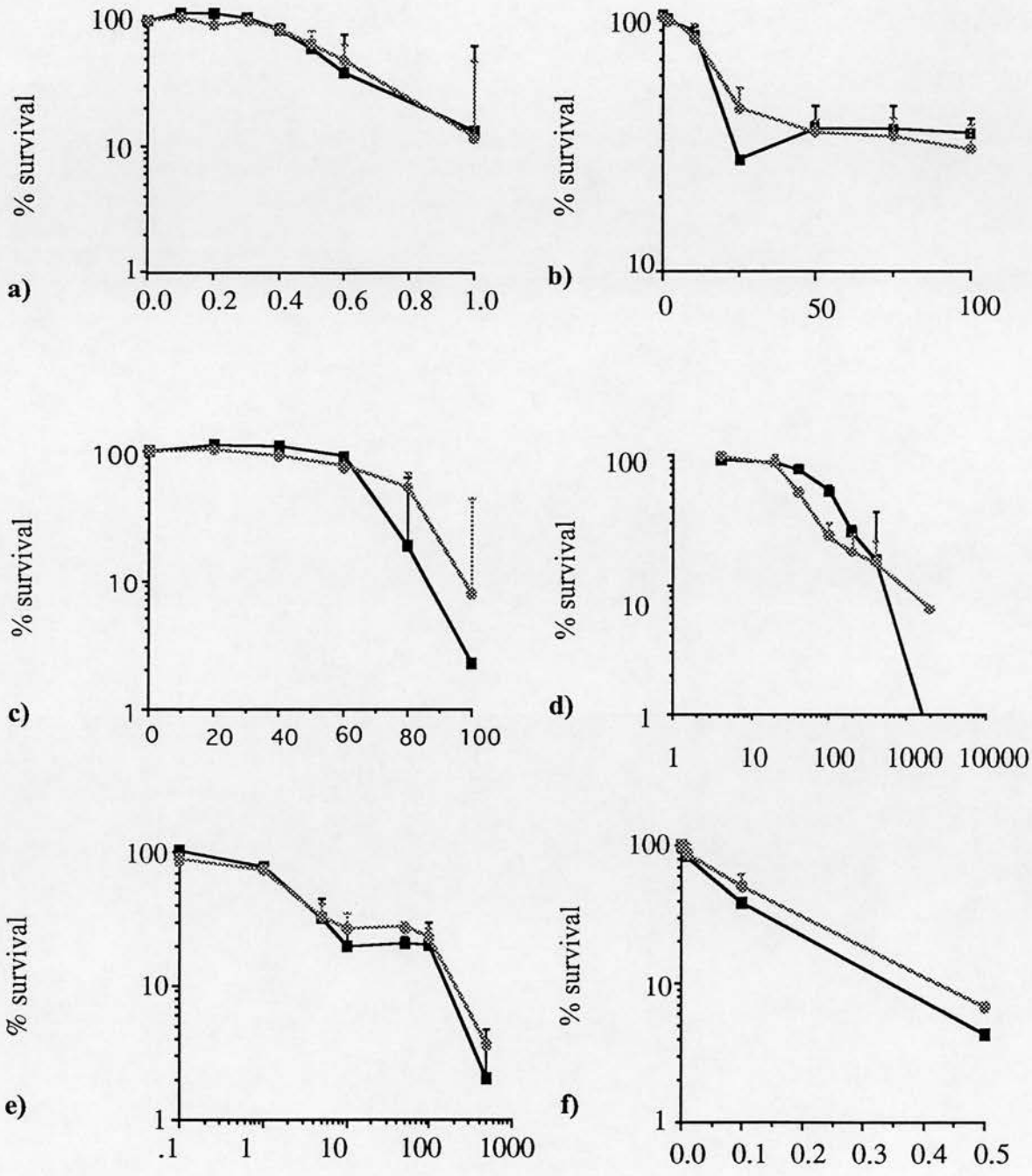


Figure 3.12 Cell survival curves based on MTT assays.

Sensitivity to the following agents was tested **a)** cadmium chloride **b)** chlorambucil **c)** ethacrynic acid **d)** paraquat **e)** bleomycin **f)** adriamycin. CHO-K1 (▪), CHO-MRc40 (⁕). All drug concentrations are μM. All data points represent the mean and standard deviation of at least triplicate samples. Where error bars are not shown they come within the area of the plot symbol.

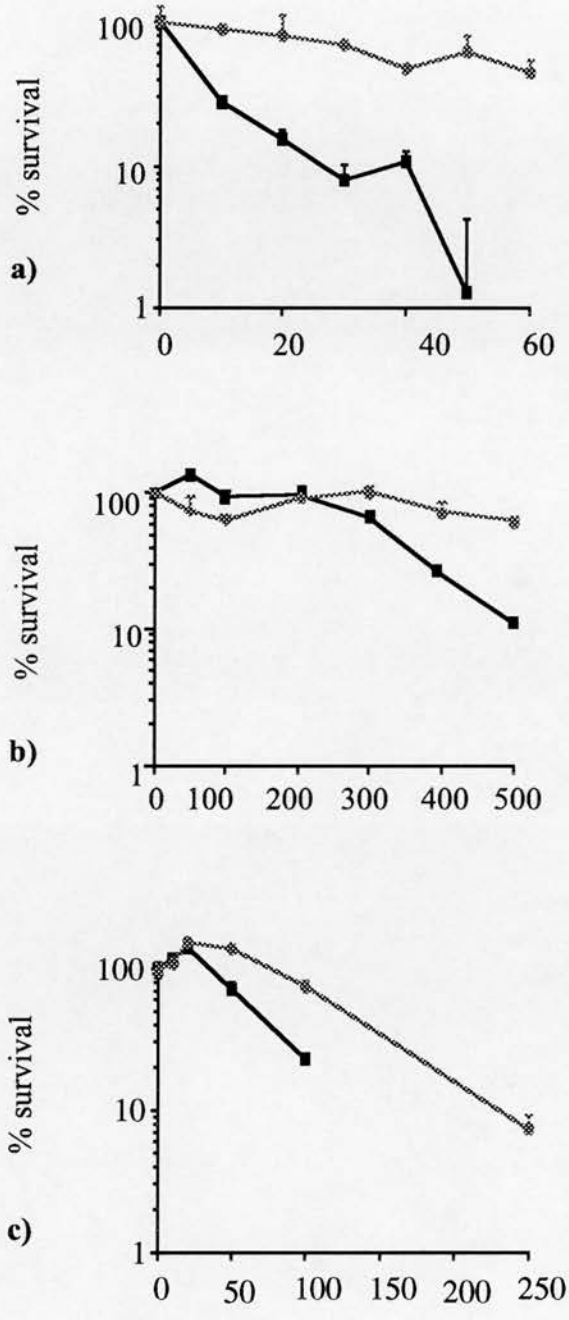


Figure 3.13 Cell survival curves based on clonogenic assays.
Sensitivity to the following agents was tested a) menadione b) hydrogen peroxide and c) sodium arsenite. CHO-K1 (■) was compared with CHO-MRc40 (❋). All concentrations are μM. Each data point represents the mean and standard deviation of three or more replicate samples. Where error bars are not shown they fall within the area of the plot symbol.

Table 3.2 *Cross resistance pattern of CHO-MRc40*

Compound	Fold resistance [a]
Menadione	**1.8**
Hydrogen peroxide	1.9
Sodium arsenite	1.6
Menadione bisulphite	1.6
Ethacrynic acid	1.3
Adriamycin	1.3
Chlorambucil	1.3
Cadmium chloride	1.1
Bleomycin	1.0

[a] $\frac{LD_{50}\ CHO\text{-}MRc40}{LD_{50}\ CHO\text{-}K1}$ (Measured by MTT assay)

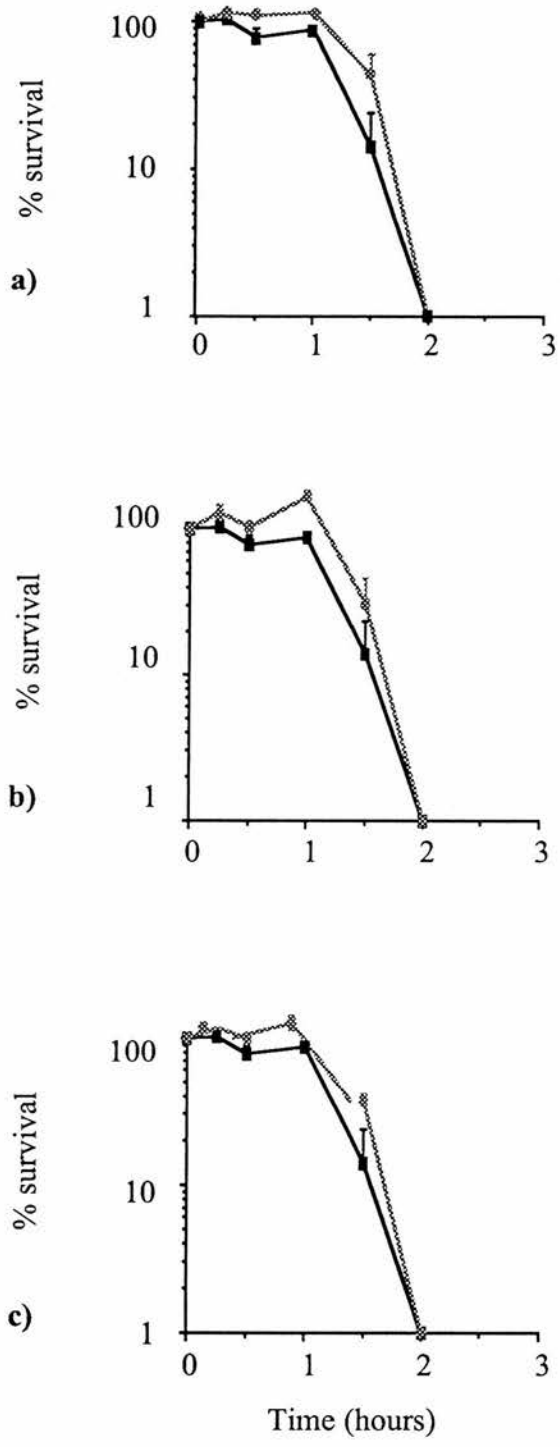


Figure 3.14 Cell survival curves following heat shock at 42° C. Survival of CHO-K1 (■) was compared with that of menadione-resistant cell lines (❋). The menadione-resistant cell lines tested were a) CHO-MRc20 b) CHO-MRc30 and c) CHO-MRc40. Each data point represents the mean and standard deviation of six replicate samples. Where error bars are not shown they fall within the area of the plot symbol.

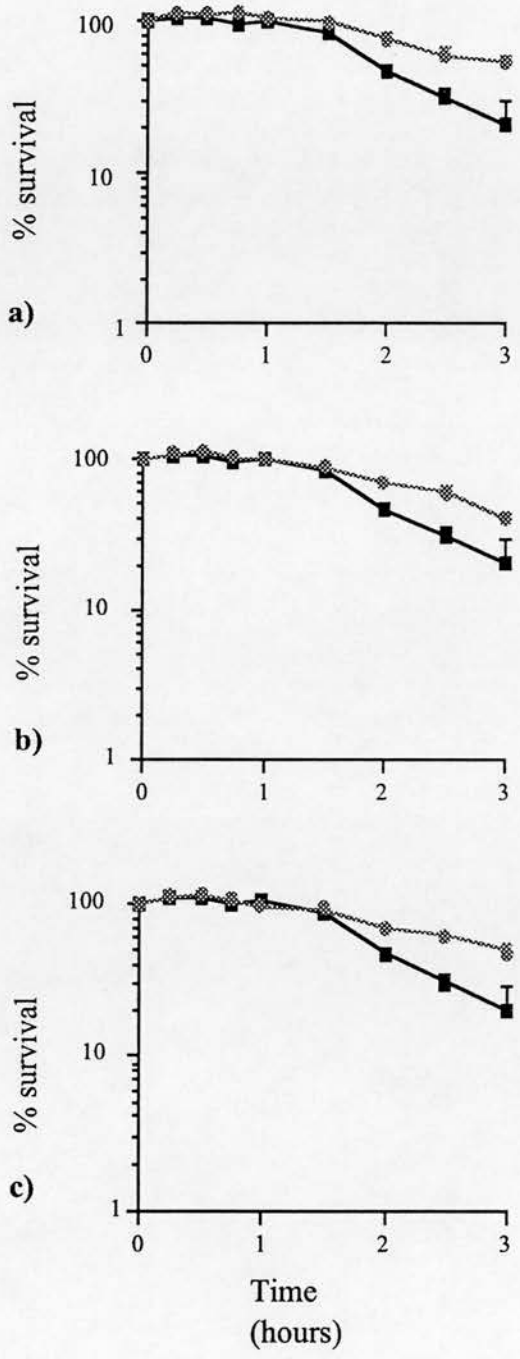


Figure 3.15 Cell survival curves following heat shock at 42° C. Survival of EJ-WT (■) was compared with that of menadione-resistant cell lines (❋). The menadione-resistant cell lines tested were **a)** EJ-MRc20 **b)** EJ-MRc30 and **c)** EJ-MRc40. Each data point represents the mean and standard deviation of six replicate samples. Where error bars are not shown they fall within the area of the plot symbol.

3.3.5 Cross-resistance to ionising radiation

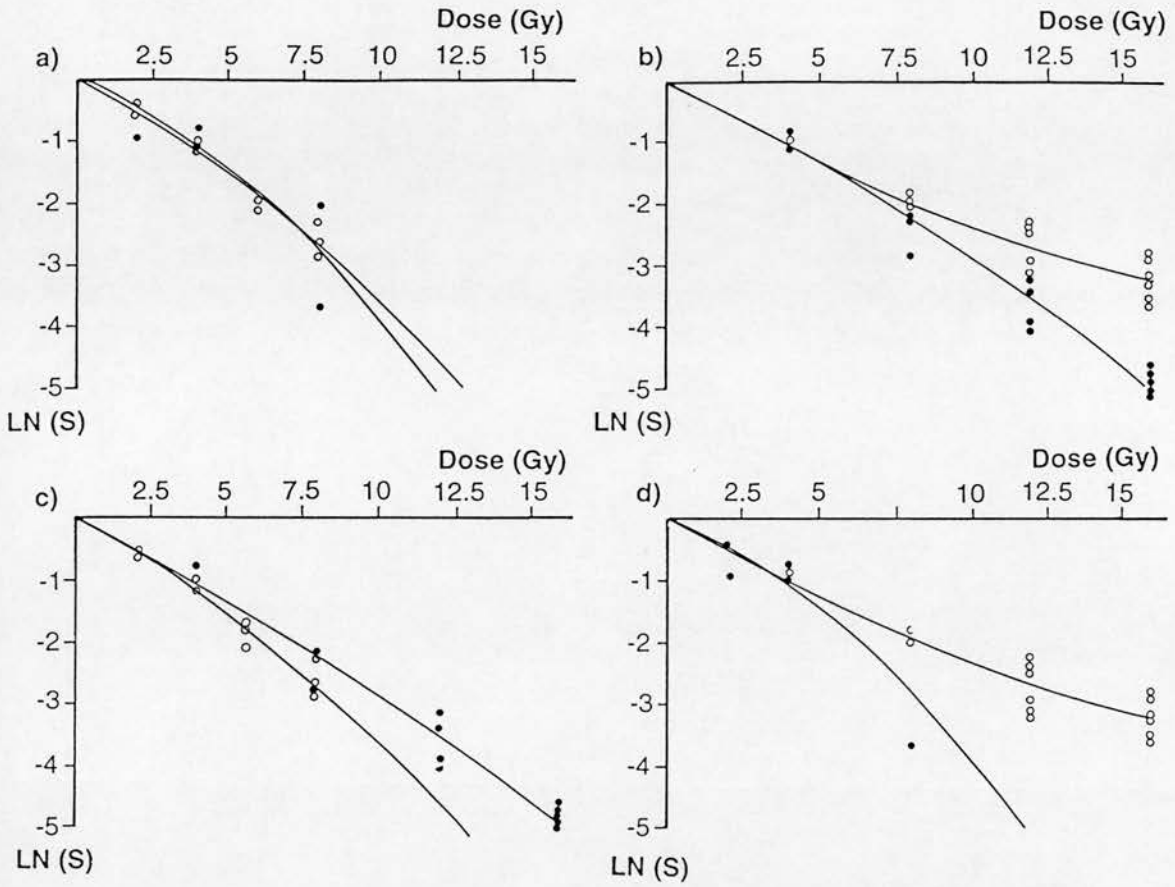


Figure 3.16 Radiation survival curves

(a) CHO-K1 (•) and CHO-MRc40 (o) in oxic conditions,

(b) CHO-K1 (•) and CHO-MRc40 (o) in hypoxic conditions.

(c) CHO-K1 in oxic (o) and hypoxic conditions (•) and

(d) CHO-MRc40 in oxic (•) and hypoxic (o) conditions.

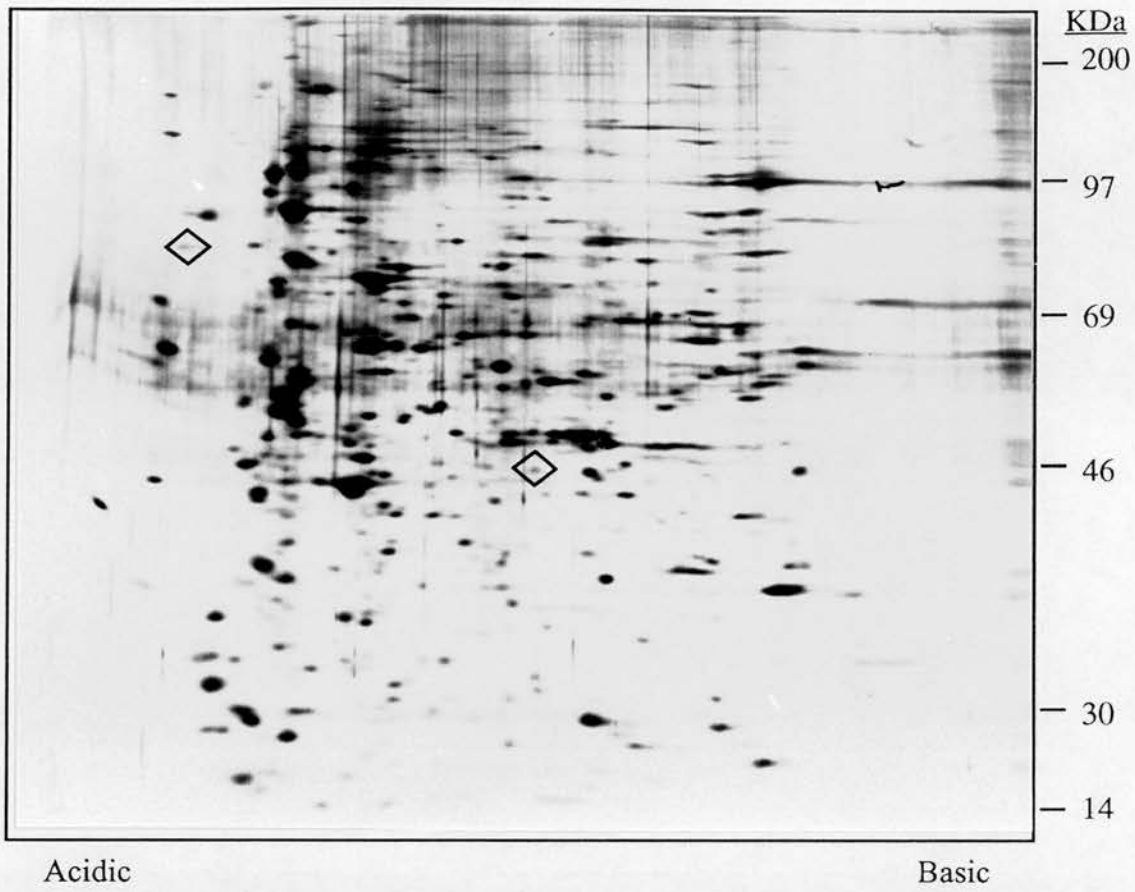
The graphs shown above demonstrate that there is no difference in the radiation response of menadione-sensitive and -resistant cells in oxic conditions (Figure 3.16 a). Both cell lines are more resistant under hypoxic compared with oxic conditions (Figure 3.16 b). The chemoresistant cell line, CHO-MRc40, is more impressively radioresistant in hypoxic conditions (Figure 3.16 d) than the parental line from which it was derived, CHO-K1 (Figure 3.16 c). There is a difference in the radiation response between the two cell lines in hypoxic conditions.

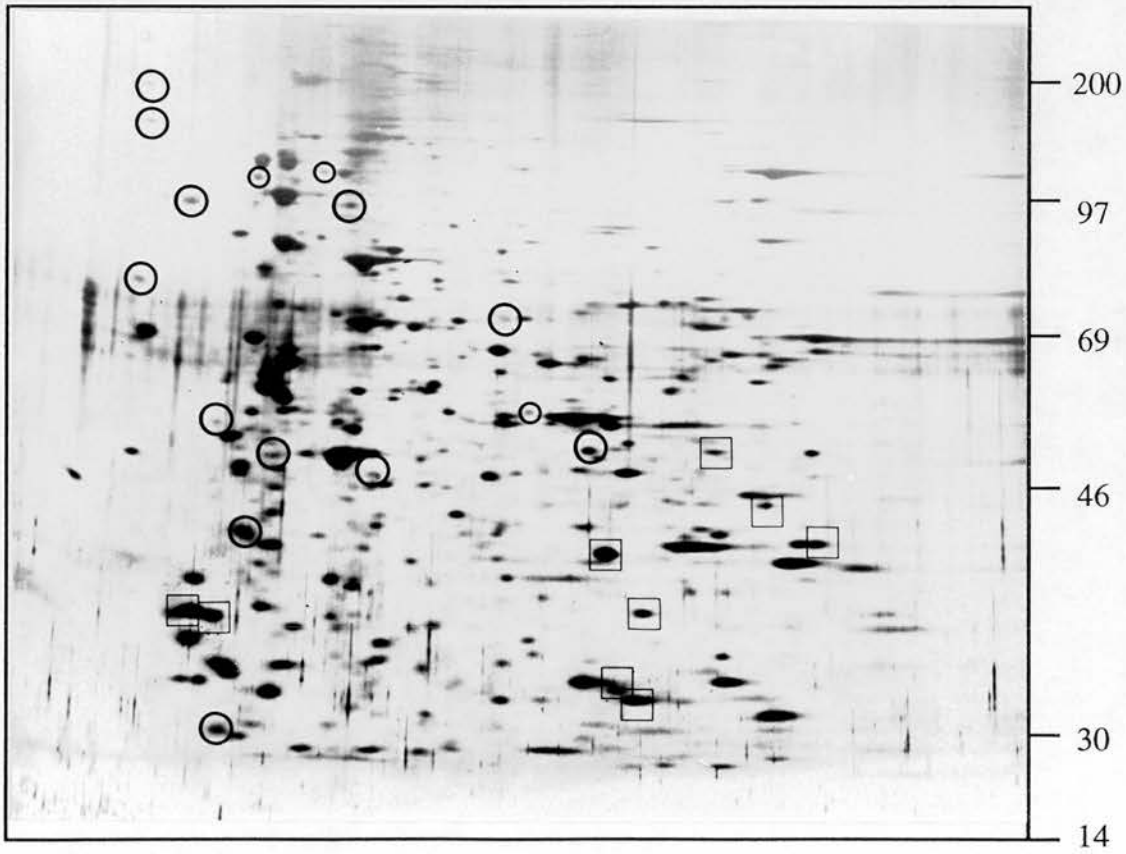
3.3.6 Analysis of proteins by two-dimensional electrophoresis

Two-dimensional gel electrophoresis was used to examine the protein composition of total cellular proteins of menadione-sensitive and -resistant cell lines. Initially the protein pattern of CHO-K1 and CHO-MRc40 lines (that had not been exposed to drug for two weeks) was compared. Gels were run as described in section 2.4.5. Proteins were visualised by silver staining (Figure 3.17). From these gels 9 proteins were identified that were induced and 15 proteins that were depressed in CHO-MRc40 compared to CHO-K1. There were also two proteins which were exclusively expressed in CHO-K1. Since CHO-MRc40 had not been exposed to menadione for two weeks prior to this experiment the observed changes must be due to permanent alteration rather than to a transitory effect of the drug. Differences may be due either to differential gene expression or to post-translational modification of protein. Similarly, when EJ-WT and EJ-MRc30 cells were pre-labelled with ^{35}S-methionine and total cell protein then used for two-dimensional gel electrophoresis a large number of alterations were observed (Figures 3.18 and 3.19). Compared with EJ-WT five proteins were elevated and 15 depressed in EJ-MRc30. Four proteins that were exclusively expressed in EJ-WT were also identified.

The nature of the response of a menadione-sensitive cell line (CHO-K1) and a menadione-resistant line (CHO-MRc40) to oxidative stress was examined by two-dimensional electrophoresis (Figure 3.20 a to d).

Figure 3.17 (See page 100) Two-dimensional gel analysis of proteins in (a) CHO-K1 and (b) CHO-MRc40 cytosolic fractions. Gels were stained with silver nitrate. The fifteen circled spots identify some of the proteins whose synthesis was reduced in the menadione-resistant cell line compared with the parental line. The nine squares identify proteins that were induced in the resistant cell line relative to the parental line. The two diamonds identify proteins that are absent in the resistant cell line. The resistant cells had not been exposed to drug for two weeks before they were harvested for this experiment.


KDa
200
97
69
46
30
14
Acidic
Basic


200
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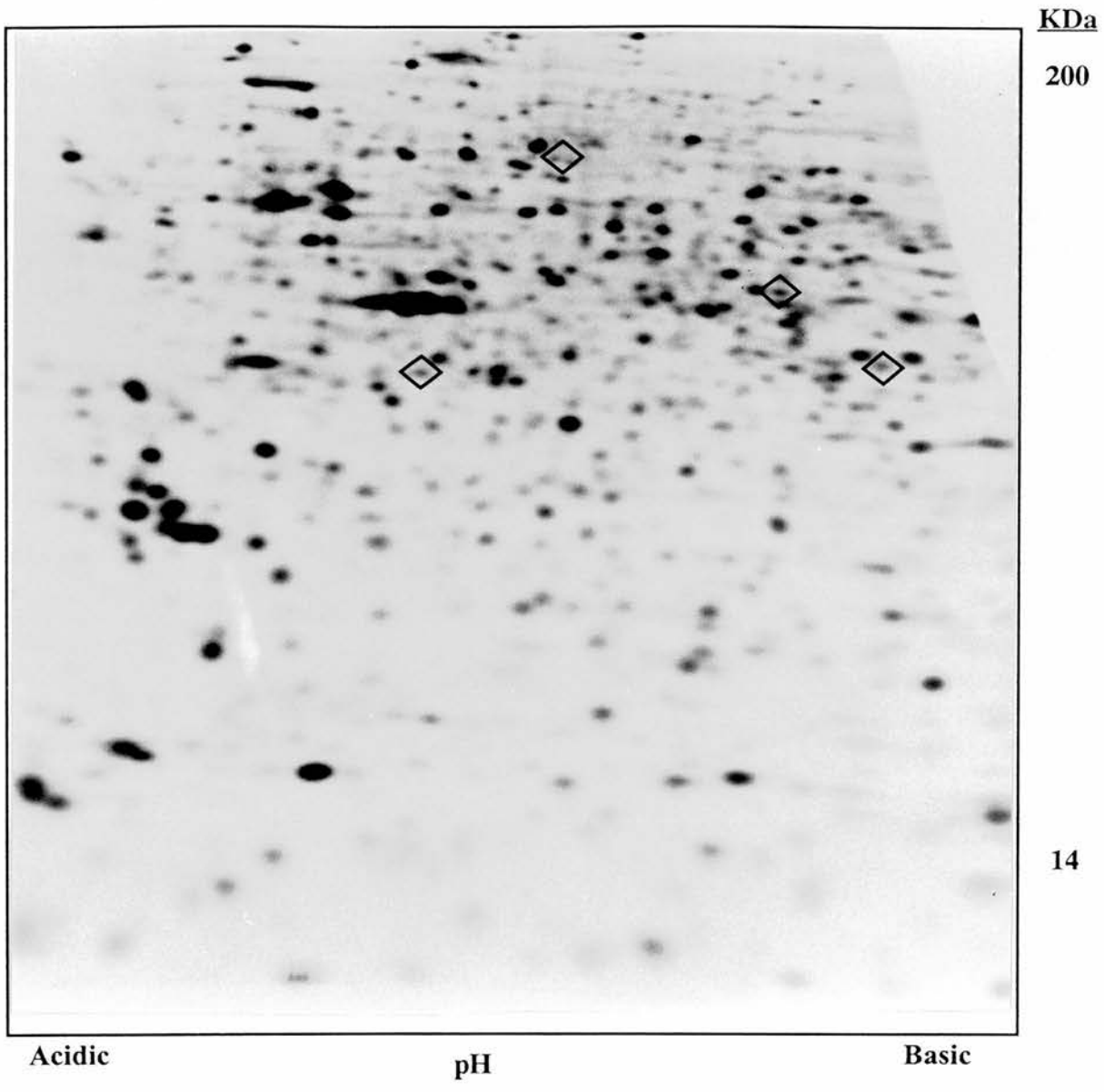


Figure 3.18 EJ-WT cell line. Analysis by two-dimensional gel electrophoresis.

Diamonds: Proteins that are expressed only in EJ-WT (see Figure 3.19 for comparison)

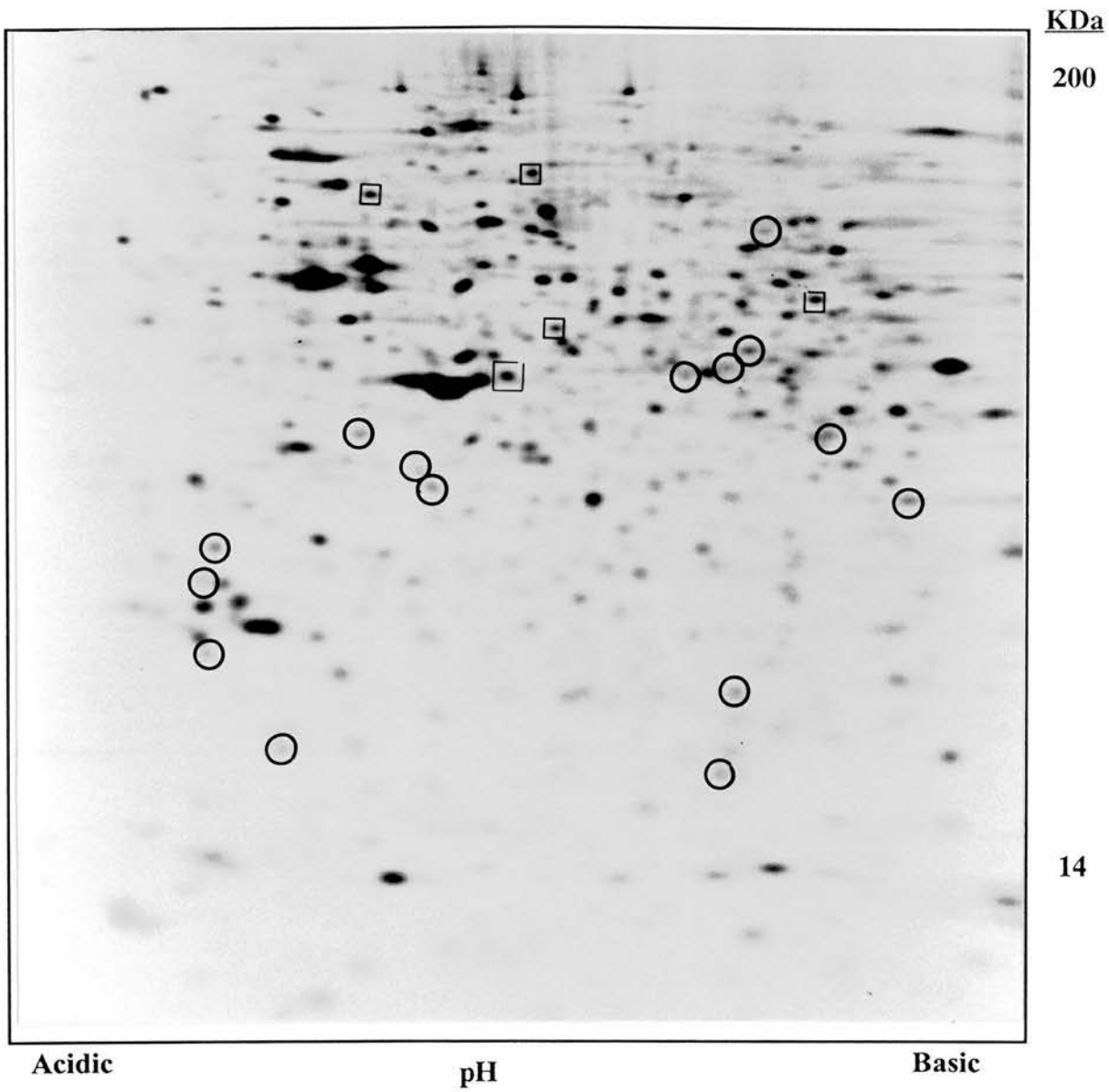


Figure 3.19 EJ-MRc30 cell line. Analysis by two-dimensional gel electrophoresis.

Circles: Proteins that are absent or diminished in EJ-MRc30 compared with EJ-WT.
Squares: Proteins that are induced in EJ-MRc30 compared with EJ-WT.
(see Figure 3.18 for comparison).

Figure 3.20

Two-dimensional gel electrophoresis of ^{35}S-methionine-labelled whole cell lysates

(a) Untreated CHO-K1,

(b) CHO-K1 treated with 500μM H_2O_2 for 30 minutes,

(c) Untreated CHO-MRc40,

(d) CHO-MRc40 treated with 500μM H_2O_2 for 30 minutes

The following symbols apply to figure 3.20 (a) to (d)

Circles: Proteins that are induced in both cell lines by oxidative stress (H_2O_2);
Squares: Proteins that are strongly induced by oxidative stress in CHO-MRc40 but not in CHO-K1.
Diamonds: Proteins that are absent or decreased in CHO-K1 compared with untreated CHO-MRc40 and compared with both cell lines following stress. The pattern of induction of these proteins suggests that they are part of an adaptive response and are constitutively induced in menadione-resistant cells.
Triangles: Proteins that are absent or diminished in CHO-MRc40 compared with CHO-K1.

This figure indicates that there are constitutive differences between menadione-sensitive and -resistant cell lines as well as differences in the pattern of protein induction/depression resulting from treatment of the cell lines with oxidants.

Figure 3.20 (a) Two-dimensional gel electrophoresis: Untreated CHO-K1

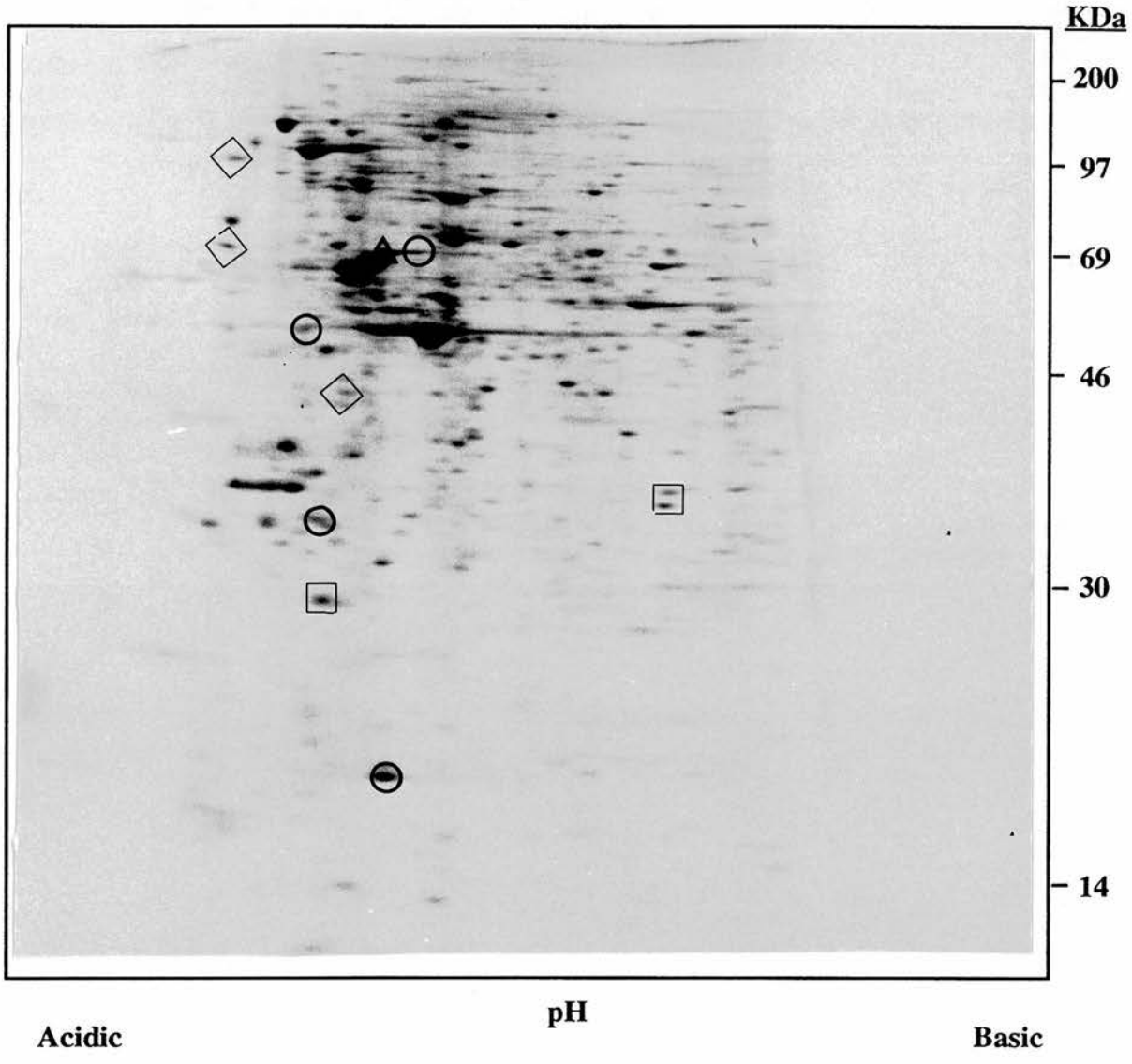


Figure 3.20 (b) Two-dimensional gel electrophoresis: CHO-K1 treated with 500μM H_2O_2.

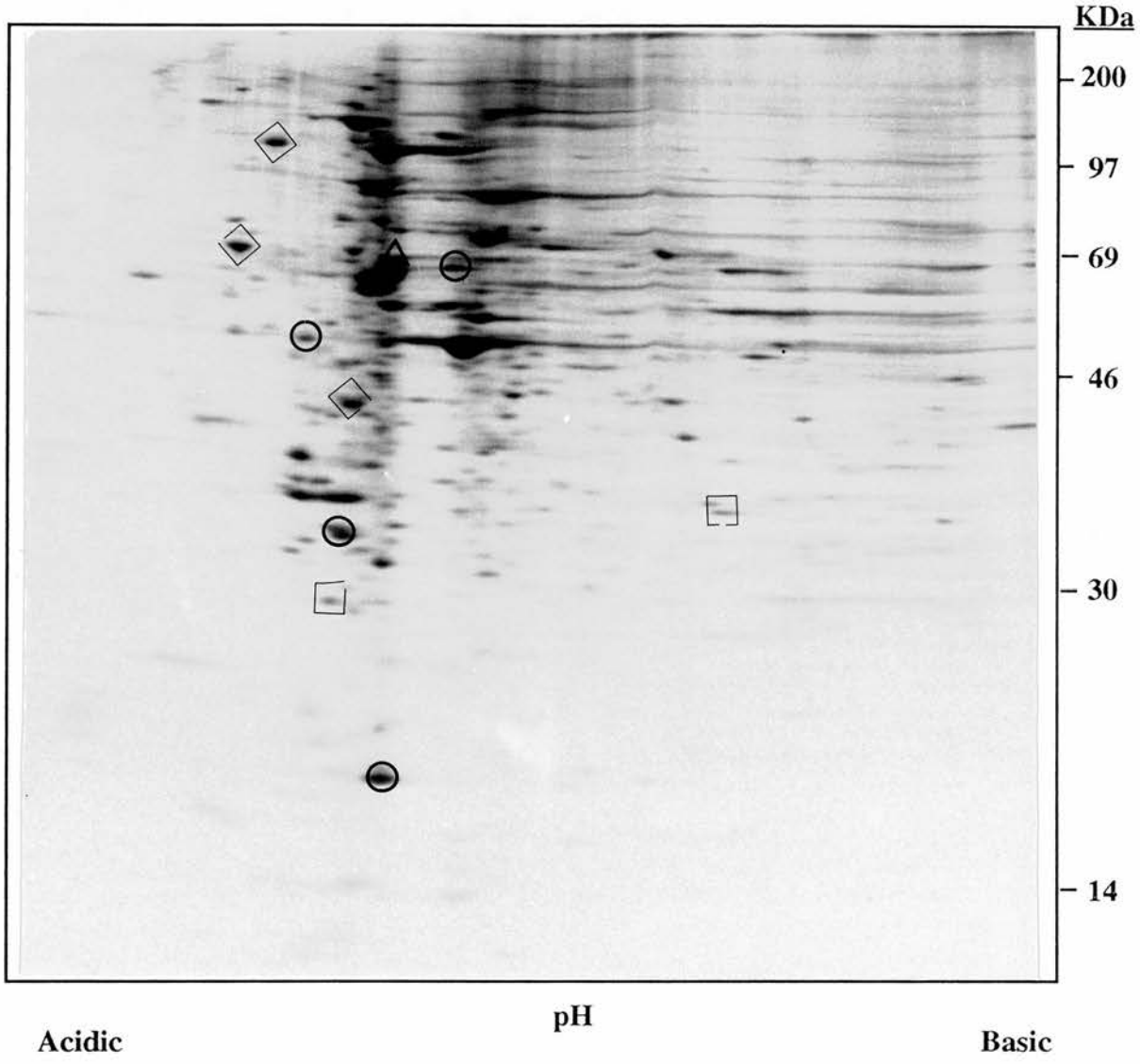


Figure 3.20 (c) Two-dimensional gel electrophoresis: Untreated CHO-MRc40

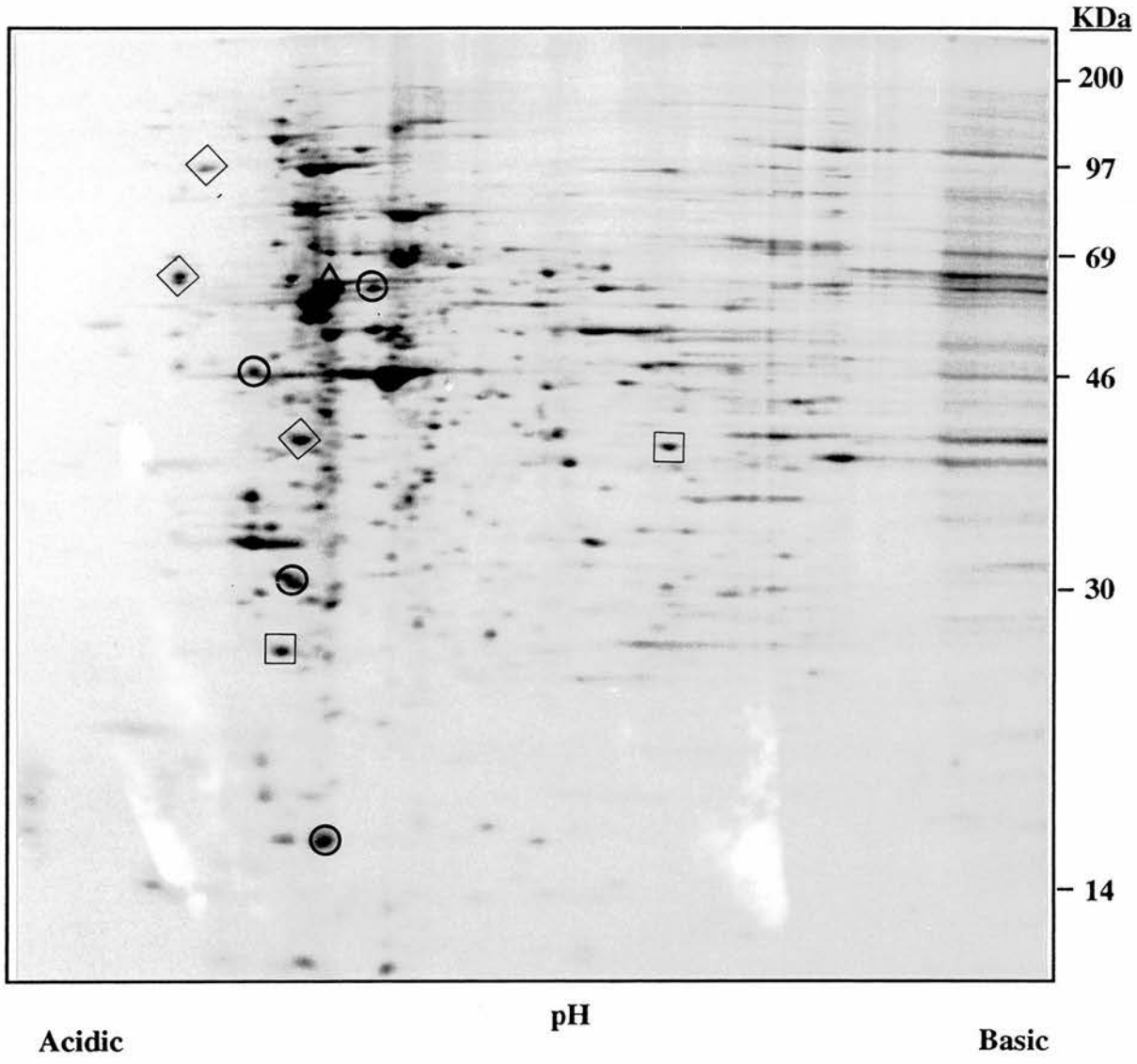
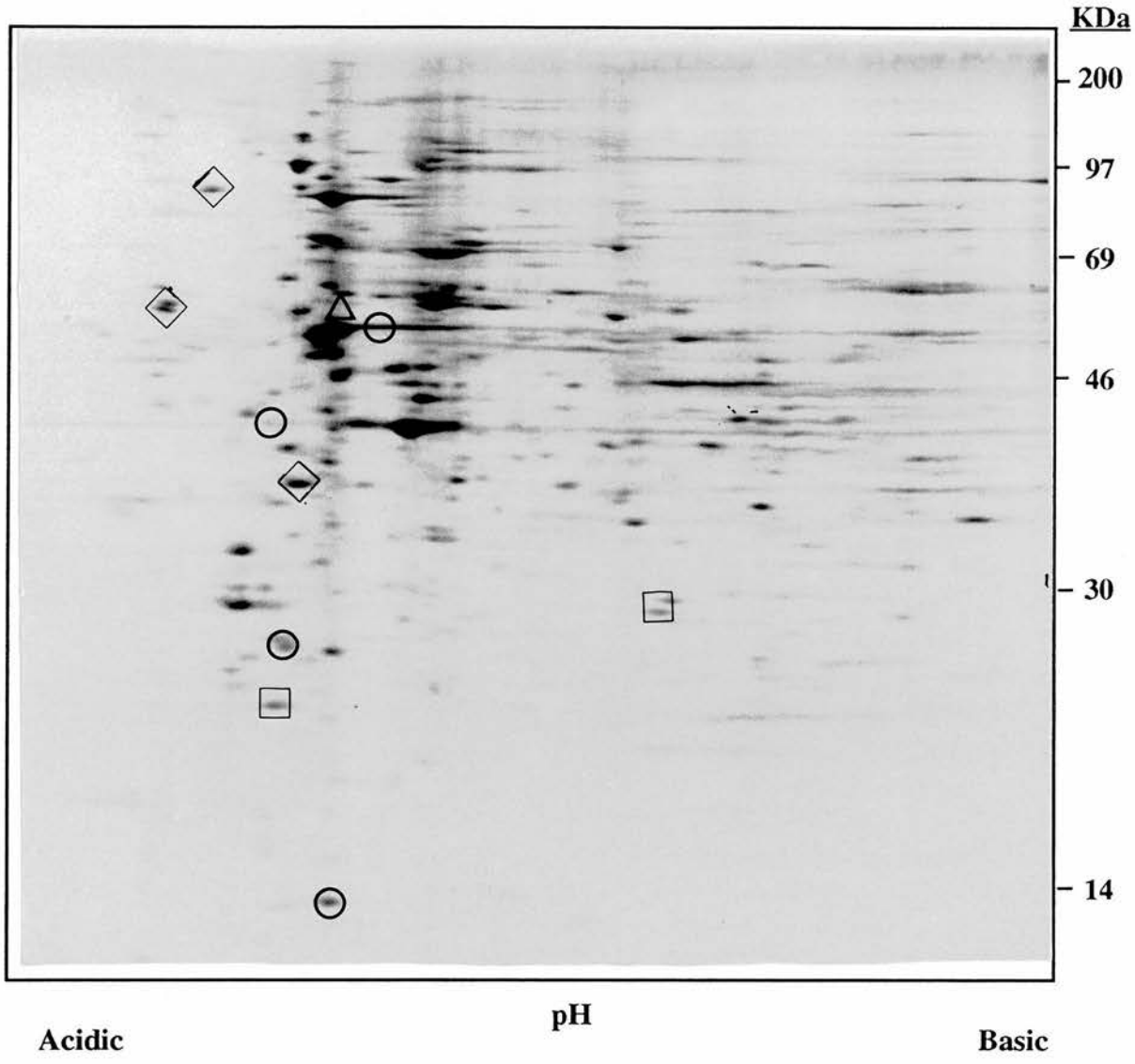


Figure 3.20 (d) Two-dimensional gel electrophoresis: CHO-MRc40 treated with 500μM H_2O_2.



3.4 DISCUSSION

As with other compounds that induce a stress response it proved very difficult to generate cell lines with a high resistance to menadione. However, after a period of forty weeks of continuous exposure to the drug, a cell line (CHO-MRc40) was isolated that exhibited up to two-fold increase in LD_{50} measured by MTT assay and 7.8-fold increase in LD_{50} measured by clonogenic assay compared to CHO-K1. In addition to resistance to menadione these cell lines exhibited cross-resistance to a variety of other oxidants and redox-cycling agents. Resistance to heat and to ionising radiation (in hypoxic conditions) was also observed.

The metabolism of menadione involves hydrogen peroxide formation and so resistance to exogenous hydrogen peroxide was predictable and indeed cross-resistance of menadione-resistant cells to hydrogen peroxide has previously been reported by Martins and Meneghini (1990). The observed resistance of menadione-resistant cells to the sulphydryl reactive agent, sodium arsenite, is interesting because the converse (resistance of arsenite-resistant cells to menadione) has also been observed. Lee and Ho (1994) recently reported that an arsenite-resistant human lung adenocarcinoma cell line (CL3R) shows two fold cross-resistance to menadione. Arsenite-resistant cells were shown to have elevated glutathione and increased HO activity and it was proposed that these features were responsible for the resistance phenotype. An earlier report by the same authors of an arsenite-resistant Chinese hamster ovary cell line again implicated raised glutathione but, also, increased GST-Pi activity in the resistance process (Lo *et al*, 1992).

The modest degree of resistance to ethacrynic acid observed in the menadione-resistant cell lines may be explained by increased expression of GST-Pi (see Chapter 5) as it is a well known substrate of this enzyme. Like menadione, adriamycin generates superoxide radical anions through redox-cycling (Sinha and Mimnaugh, 1990) and it is therefore not surprising that the menadione-resistant cells show cross-resistance to this agent, albeit rather modest. Cross-resistance to paraquat might also have been predicted since it, too, is a redox-cycling agent. Interestingly, it has been reported that there was a lack of cross-resistance to menadione by a paraquat-resistant Chinese hamster ovary cell line (Nutter *et al*, 1991). Resistance to bleomycin might also have been expected particularly as the menadione-resistant cells are resistant to radiation and bleomycin is considered to be radiomimetic in its actions. Although bleomycin exerts some of its toxic effects through intracellular generation of reactive oxygen species and DNA damage, this is not be the only mechanism by which it kills cells. The finding that menadione-resistant cells are cross-resistant to agents that

induce oxidative stress indicates that protection against oxidative stress and not reduction of menadione uptake or accumulation is the primary resistance mechanism. Menadione-resistant cells tended to revert to wild type phenotype when drug was removed from the growth medium but the CHO-MRc40 line still showed 1.3 fold resistance to menadione after 3 months of growth in drug-free medium.

Menadione-resistant cell lines isolated from both CHO-K1 and EJ-WT exhibited a modest degree of cross-resistance to heat. The exact mechanisms by which heat kills cells are unknown although possible targets for hyperthermic injury are cell membranes, cytoskeletal structures, energy metabolism, protein synthesis and DNA. Whatever the critical target of heat-induced damage there is evidence that it is mediated by free radicals. If the hypothesis that heat damage is mediated by free radicals is true, then the redox state of the cell at the time of heating might be expected to influence the degree of damage inflicted. There is evidence that this is indeed the case. Several cell culture studies show that glutathione depletion by buthionine sulphoximine significantly enhanced thermosensitivity of cells (Mitchell and Russo, 1983; Freeman *et al*, 1985). Steels *et al* (1992) found that the pretreatment of ten human tumour cell lines with buthionine sulphoximine synergistically increased the toxicity of heat and hydrogen peroxide. It is possible that proteins with active SH groups that depend on GSH for maintenance of the reduced form might be common targets for both heat and oxidative stress, hence the synergy of these two agents. Conversely the elevation of intracellular glutathione had a protective effect. The survival of HeLa cells subjected to heating at 43.5°C for 3 hours increased from 17% to 64% of controls when heating was preceded by incubation of the cells with glutathione (100μM) for eighteen hours. Burdon *et al* (1987) reported that hyperthermia resulted in the inhibition of protein synthesis in HeLa cells but that this effect was diminished in the presence of sodium azide or mannitol. Since both of these agents are capable of scavenging free radicals, the observed inhibition may have been due to the damaging effects of oxygen-derived free radicals. Donati *et al* (1990) found that oxidative stress treatments such as hydrogen peroxide, UV radiation, sodium arsenite and cadmium chloride induced synthesis of heat shock proteins in human cells. These observations suggest that oxygen radicals and cellular antioxidant defences may play an important role in the pathogenesis of hyperthermic injury and may share common mechanisms of induction and tolerance with oxidative stress. It has been suggested that elevation of glutathione may actually be a feature of thermotolerance. Lilly *et al* (1986) found that human fibroblasts treated with hyperthermia to induce thermotolerance resulted in a 3-fold elevation of glutathione. However, thermotolerance was also inducible in

fibroblasts with constitutively decreased glutathione synthesis suggesting that heat-induced elevation in glutathione is not necessary for the development of thermotolerance. One of the important changes in menadione-resistant cell lines compared with the parental lines from which they were isolated is that their intracellular glutathione concentration is elevated (see Chapter 4). This may account for the observed modest degree of cross-resistance to heat.

There is much interest in the role of thiols and redox enzymes as modulators of radioresponsiveness (Astor, 1984; Jensen and Meister, 1983). Ionising radiation produces hydroxyl radicals in aqueous solutions by direct radiolytic attack on water (Blok and Loman, 1986). The commonly accepted hypothesis for the radioprotective effect of glutathione is that it acts as a radical scavenger and is capable of hydrogen donation (Johansen and Howard-Flanders, 1965). This hypothesis would predict that increased levels of thiol compounds like GSH should provide enhanced protection from X-rays. However, the intracellular glutathione concentration has only a slight or undetectable effect on radioresponsiveness in oxic conditions. When cells are irradiated in the presence of oxygen, radiation-induced radicals produced in critical molecules (such as DNA) undergo reactions with either oxygen leading to fixation of damage and cell death or with reducing species (such as glutathione) which leads to damage repair (for example, through H-atom donation) and cell viability (Alper and Howard-Flanders, 1956). Thus damage by ionising radiation is enhanced by oxygen (Dewey, 1960). The differences in rate constants between fixation of radical damage by oxygen and repair by reducing agents predicts that species such as glutathione will have little or no effect on aerobic cell radiosensitivity. Russo and Mitchell (1984) studied the radiation response of Chinese hamster ovary cells after elevation of intracellular glutathione levels. Elevation of glutathione was achieved by exposing them to 2-oxo-thiazolidine-4-carboxylate (OTZ). OTZ is a compound with a latent thiol group as part of its ring structure - that is enzymatically opened - and then serves as an intracellular cysteine delivery system and so promotes glutathione synthesis. It was found that elevation of glutathione by as much as 200-300% provided no protection in air. Furthermore, the depletion of glutathione to less than 5% of normal led to only slight sensitisation of the aerated radiation survival response.

Another approach to assessing the influence of glutathione on radioresponsiveness has been to compare the radiation survival of cells with variable glutathione content. For example, Carmichael *et al* (1988a) compared the radiosensitivity of a number of colorectal cell lines. Variable sensitivity to X-rays was observed but there was no correlation with the glutathione content of the cells (which varied from 1.3 to 207nmol/mg protein). There have been a few reports of

radioprotection of cells by increasing intracellular glutathione (Jensen and Meister, 1983).

Although glutathione plays only a limited role in determining the aerobic radiation response, it appears to have a more profound effect in hypoxic conditions. There is considerable evidence to support the contention that depletion of intracellular glutathione sensitises hypoxic cells to ionising radiation (Bump *et al*, 1982; Biaglow and Varnes, 1983). Under hypoxic conditions metabolically reduced intermediates react with glutathione instead of oxygen and so the availability of thiols becomes critical in determining the net damage to the cell. The glutathione concentration of CHO-MRc40 is twice that of CHO-K1. This may explain the increased radioresistance of the CHO-MRc40 cell line in hypoxic conditions.

3.5 SUMMARY

Stable variants of cultured mammalian cells resistant to the naphthoquinone compound, menadione, have been isolated. The most successful strategy was continuous exposure of cells to the selective agent. Initial characterisation indicated that menadione-resistant cell lines were cross-resistant to a number of chemical and physical agents which cause oxidative stress. The resistant phenotype was maintained after removal of the selective agent although there was a tendency for cells to revert to wild-type phenotype. Protein gel electrophoresis indicates that there was a marked difference in protein composition of wild-type and resistant cell lines since the resistant cells had not been exposed to drug for 2 to 3 weeks. These changes could not be attributed to an acute response to stress but to permanent alteration in the genotype.

CHAPTER FOUR

GLUTATHIONE METABOLISM IN MENADIONE-RESISTANT CELL LINES

4.1 INTRODUCTION

In the preceding chapter the successful isolation of menadione-resistant cells was described. The next step was to analyse the cell lines to establish the molecular mechanisms contributing to the resistance phenotype. Glutathione metabolism was an obvious aspect of cell metabolism to investigate first. This is because glutathione has frequently been implicated in acquired drug resistance as well as in the cellular response to radiation and heat.

4.1.1 The interaction of menadione and glutathione

The addition of menadione to cells is invariably followed by the depletion of glutathione. As the concentration of reduced glutathione falls so the cell is less able to deal with free radicals which therefore accumulate and cause cytotoxicity. Several different mechanisms contribute to the loss of reduced glutathione. Di Monte *et al* (1984a) studied the effect of menadione metabolism on freshly isolated rat hepatocytes and demonstrated a dose-dependent decrease in glutathione by three mechanisms. Firstly, oxidation of GSH to GSSG took place and accounted for 75% of the loss. Secondly, there was direct reaction of menadione with glutathione to form a glutathione-menadione conjugate and this accounted for 15% of the loss. Lastly the formation of glutathione-protein mixed disulphides took place.

One of the products of the redox-cycling process, hydrogen peroxide, interacts with reduced glutathione in the presence of glutathione peroxidase, to produce water and disulphide glutathione. Large amounts of GSSG are formed by the glutathione peroxidase system. This is then either reduced back to glutathione by glutathione reductase or excreted from the cell into the medium. In the hepatocyte model used by Di Monte the intracellular concentration of GSSG reached a maximum at 5 minutes and returned to normal by 30 minutes. The concentration of GSSG in the medium was by this time much higher than pretreatment.

There is experimental evidence that menadione interacts directly with glutathione to produce a menadione-glutathione conjugate (Ross *et al*, 1985). Menadione reacts directly with GSH by Michael addition and this reaction results in formation of the menadione-glutathione conjugate, 2-methyl-3-glutathionyl-1,4-naphthoquinone (thiodione). Nickerson *et al* (1963) were able to demonstrate the *in vitro* formation of this conjugate by mixing solutions of menadione and glutathione in 70% ethanol. The reaction products were precipitated, purified and examined by paper chromatography. During the course of this reaction, sulphydryl groups were depleted. The amount of thiodione formed and SH lost were in good agreement at all stages of the reaction. Glutathione was presumed to attach to the quinone ring via an SH group. Di Monte *et al* (1984b) used a more sophisticated technique to demonstrate the formation of a menadione-glutathione conjugate. They mixed a solution containing both menadione and glutathione and after incubation this was run on an HPLC column. A peak which was not due to either menadione of glutathione appeared on the HPLC trace. When either ^{3}H-menadione or ^{3}H-glutathione were used the eluate corresponding to this HPLC peak - contained a significant amount of radioactivity. That is to say the reaction product was a glutathione conjugate of menadione. In another experiment to confirm these findings, hepatocytes that had been depleted of glutathione with BSO were treated with ^{14}C-glycine. The radioisotope was incorporated into newly synthesised glutathione. ^{3}H-menadione was added. Now the HPLC peak corresponding to the menadione-glutathione conjugate contained significant quantities of both ^{14}C and ^{3}H. The amount of conjugate formed depended on the initial concentrations of intracellular glutathione and menadione. The rate constant for the reaction of menadione with glutathione was 10 $M^{-1}s^{-1}$. The conjugate has itself been shown to redox-cycle in isolated, perfused rat liver and in microsomal suspension with supplementary NADPH (Wefers and Sies, 1983). The conjugation reaction between menadione and glutathione does not disturb the quinone nucleus, and it is for this reason that the menadione-glutathione conjugate retains the capacity to redox-cycle.

A major source of cellular thiols are those contained in proteins and menadione may interact with these SH groups. There is a marked decrease of protein sulphydryl during menadione metabolism. After the addition of ^{3}H-menadione to hepatocytes, covalently bound radioactivity was found to be associated with cellular proteins (Di Monte *et al*, 1984b). When a high dose of menadione (200μM) was used the amount of protein-bound menadione was continuing to increase even at 120 minutes. That protein binding was specifically to SH groups was shown by first incubating hepatocytes with the thiol-complexing agent - *N*-ethylmaleimide (NEM). No

appreciable binding of menadione to protein occurred in cells or microsomes pretreated with NEM. The binding of menadione to sulphydryl groups in protein is non-enzymatic because it occurs in the absence of NAD(P)H. Most loss of protein thiol groups appears to be due to oxidation. This was demonstrated by reducing protein samples with $NaBH_4$ (50mg/ml). This agent restored the bulk of the protein thiol pool - without significantly affecting the amount of bound menadione. It was calculated that one sixth of protein thiols lost during menadione metabolism was due to binding of menadione and the remainder to oxidation.

4.2 STRATEGIES

The organic sulphydryl (SH) imparts nucleophilicity to low molecular weight compounds such as cysteine and glutathione and to the cysteine residues of proteins. The oxidation of SH compounds gives rise to a number of disulphide forms including low molecular weight compounds such as cystine (cys_2) and glutathione disulphide (GSSG), mixed adducts between low molecular weight thiols and protein thiols and also disulphides within proteins themselves. Glutathione and low molecular weight thiols can be analysed by a variety of enzymatic, spectrometric and chromatographic approaches (Meister and Anderson, 1983). HPLC was used in this study because it is accurate, sensitive and allowed the simultaneous measurement of free low molecular weight thiols and total thiols. The term "free thiols" includes reduced low molecular weight thiols (particularly GSH and CysH) as well as bromobimane accessible protein thiols. To measure "total thiols" samples are reduced with dithiothreitol (DTT) which renders oxidised low molecular weight thiols (GSSG and cys_2) and oxidised protein thiols accessible to derivatization with monobromobimane.

Following an oxidative insult it is the ability to rapidly replenish reduced glutathione rather than the absolute concentration of glutathione that determines the amount of damage to the cell. Rather than simply measure and compare the absolute concentration of glutathione in the cells we also carried out experiments to determine the kinetics of glutathione metabolism in menadione-sensitive and resistant cells.

NMR spectroscopy was first used to study whole cell suspensions during the 1970s (Brindle *et al*, 1979). It is a technique which enables specific metabolites to be monitored in the living cell and has been used to study a range of intact systems including muscle and bacteria (Lapidot and Irving, 1977). A spin-echo pulse sequence can be used that takes advantage of the differences in relaxation times between large and small molecules in solution. It is therefore possible to tune out the signal arising from large molecules and to produce a spectrum composed solely of

small molecules of which glutathione is an important example. NMR has proven useful in the non-invasive measurement of the thiol redox status (Livesey *et al*, 1989) as changes in the GSH/GSSG ratio are easily visualised (McKay *et al*, 1986; Al-kaban *et al*, 1988). The resonances due to glutathione are shown in Figure 4.6. The g2 signal arises from the cysteinyl residue of glutathione and g4 arises from the glutamyl residue. When glutathione is in the reduced state g2 is negative and larger than g4 but in the oxidised state it is smaller. Thus the proportion of glutathione in the reduced state can be estimated for any time point.

4.3 RESULTS

4.3.1 High Performance Liquid Chromatography

(a) CHO cell lines

HPLC was used to measure both free and total low molecular weight thiols in CHO-K1 and CHO-MRc40 following the addition of menadione (Figures 4.1 and 4.2). The resting concentration of total glutathione in CHO-K1 and CHO-MRc40 was 6.0 and 12.1 nmol/mg protein respectively (Figure 4.1). There was therefore a 2-fold increase in glutathione in the menadione-resistant compared with the menadione-sensitive cell line. On exposure to menadione, the level of glutathione falls in both cell lines. However, depletion of glutathione is more profound and its recovery is delayed for longer in CHO-K1 compared with CHO-MRc40. In CHO-MRc40 the glutathione concentration returned to the unstressed level by 6 hours but in the CHO-K1 line this took 24 hours. Furthermore, the rise in glutathione concentration in the menadione-resistant cells far exceeds the pretreatment value. The maximum concentration of total glutathione in the menadione-resistant cell line following treatment with menadione was 26.3nmol/mg protein, which occurred at 12 hours. This is a 2.2-fold increase in glutathione compared with the same cell line in the unstressed state. The level of glutathione in the parental cell line never rose significantly above the pretreatment level during the recovery period. Thus by 12 hours after the administration of menadione the concentration of free glutathione in CHO-MRc40 cells is 9.7-fold greater than that of CHO-K1. It would have been interesting to continue the experiment and measure levels at later time points. However, this was not possible because after 24 hours in the presence of menadione a large percentage of the wild type cells were dead.

A similar pattern of initial depletion followed by recovery is also seen with

intracellular cysteine. The resting concentration of total cysteine in CHO-K1 and CHO-MRc40 was 2.5 and 7.9 nmol/mg protein respectively. There was, therefore, a 3.2-fold increase in cysteine in the menadione-resistant compared with the menadione-sensitive cell line. As with glutathione, the level of cysteine achieved during the recovery phase is greater for CHO-MRc40 than for CHO-K1. The maximum concentration of total cysteine in the menadione-resistant cell line following treatment with menadione was 10.5 nmol/mg protein (at 24 hours). This is a 1.3-fold increase in cysteine compared with cells in the unstressed state and a 4-fold increase in cysteine compared with CHO-K1 at the same time point.

The difference between the total and free thiol concentration values is a measure of the amount of thiol present in the oxidised form. In Figure 4.3 the data from the experiment shown in figures 4.1 and 4.2 is replotted to allow comparison of the concentrations of free and total glutathione and cysteine. In the case of glutathione it can be seen that the free and total thiol concentrations are very similar and this remains true following the addition of menadione. In the case of cysteine the total concentration significantly exceeds the free concentration. These experiments therefore demonstrate that the redox status of glutathione lies in favour of the reduced form whereas that of cysteine lies more in favour of the disulphide species. This is true of both menadione-resistant and -sensitive cell lines.

(b) EJ cell lines

The pattern of the response of cellular thiols to treatment with menadione in EJ cell lines is shown in Figures 4.4 and 4.5. The pretreatment levels of total glutathione for the wild-type and menadione-resistant cell lines were essentially the same being 28.2 and 27.0 nmol/mg protein respectively. Although the starting total concentration of glutathione was the same for the two cell lines a greater percentage of glutathione was in the reduced form in menadione-resistant than -sensitive cells (86% and 64% respectively). The resting concentration of total cysteine in EJ-WT and EJ-MRc30 was 2.5 and 2.8 nmol/mg protein respectively. Although again a greater percentage of the thiol was in a reduced form in EJ-MRc30 compared with EJ-WT (86% and 68% respectively).

The profundity of the fall in glutathione and cysteine levels following the addition of menadione was not so extreme as that seen in the CHO cell lines. This was probably because a much lower concentration of menadione was used in this experiment (7.5μM) compared with that used for the CHO cell lines (25μM). The EJ-WT were more sensitive to menadione than CHO-K1 and had a higher dose of the

drug been used there would have been insufficient viable cells left at the later time points to allow the HPLC analysis to be performed. However, the rapid and efficient replenishment of glutathione in the drug-resistant cell line is seen in the EJ cells as it was in the CHO cells.

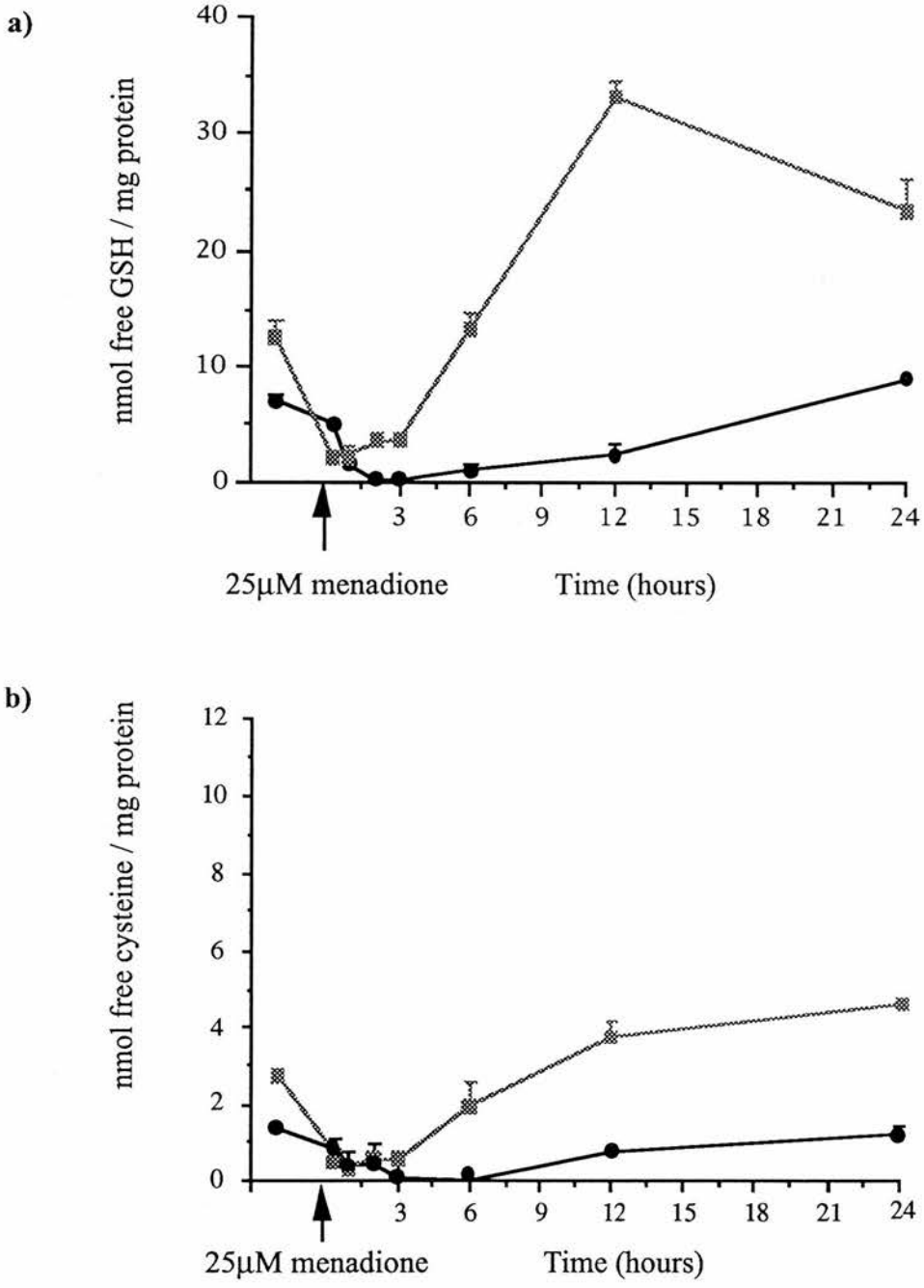


Figure 4.1 Time course of **(a)** free glutathione and **(b)** free cysteine concentration in CHO-K1 (●) and CHO-MRc40 (▩) following the addition of 25μM menadione to culture medium. Each data point represents the mean and standard deviation of duplicate measurements. Where not shown, error bars fall within the area of the plot symbols. Results are from one representative experiment.

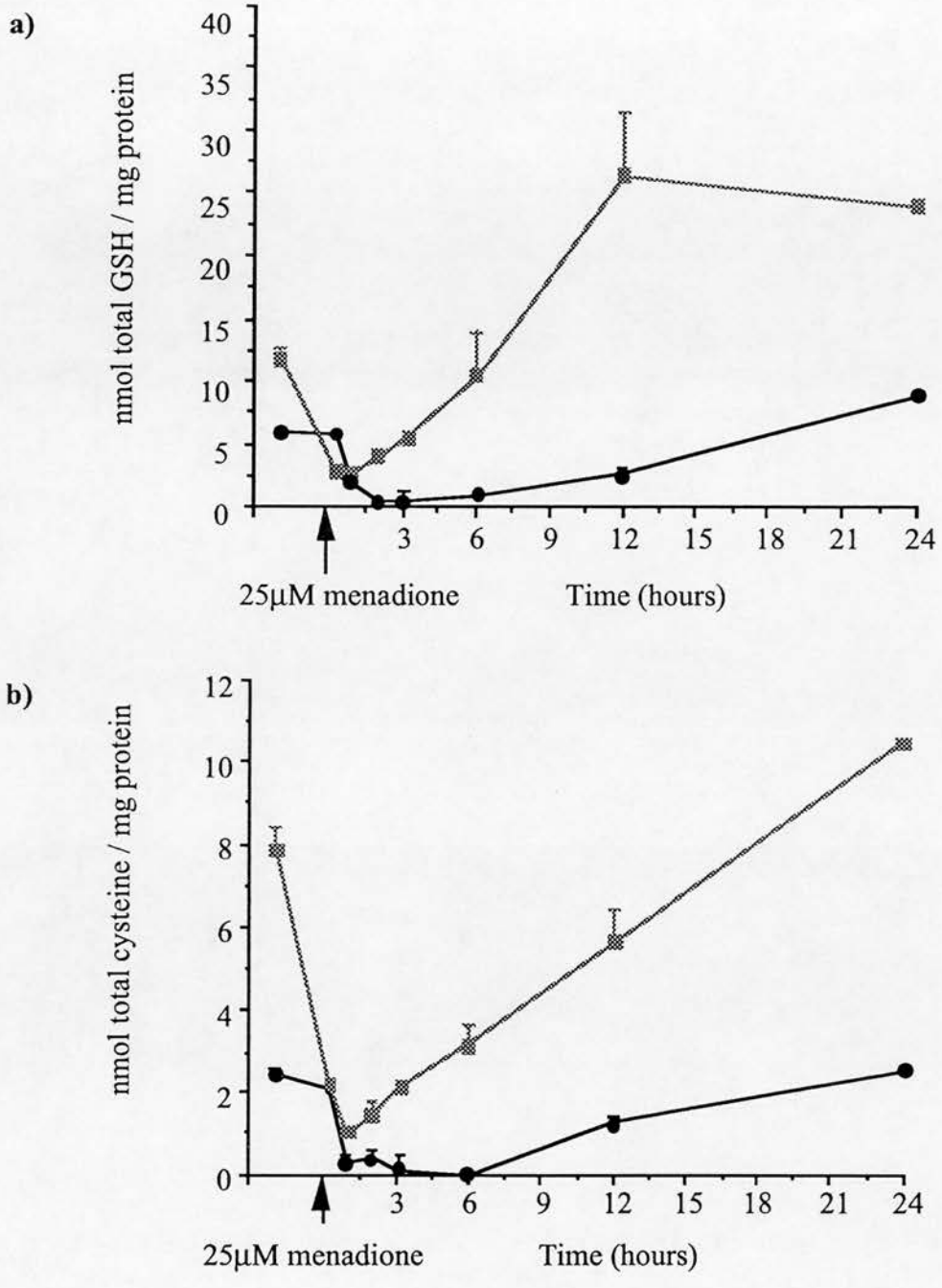


Figure 4.2 Time course of **(a)** total glutathione and **(b)** total cysteine concentration in CHO-K1 (●) and CHO-MRc40 (▩) following the addition of 25μM menadione to culture medium. Each data point represents the mean and standard deviation of duplicate measurements. Where not shown, error bars fall within the area of the plot symbols. Results are from one representative experiment.

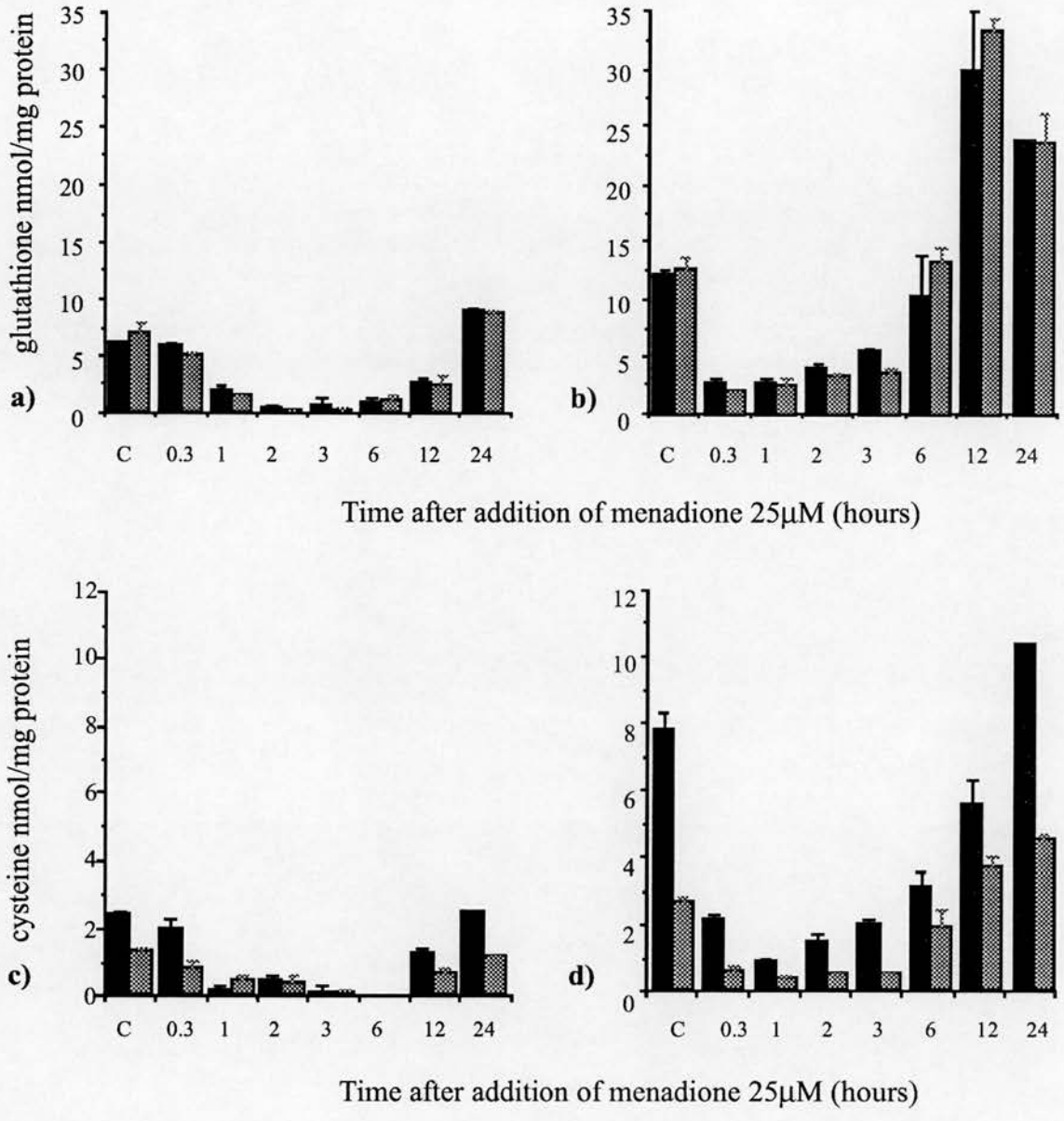


Figure 4.3 Bar charts showing relative concentrations of total ■ and free ▩ low molecular weight thiols in CHO cell lines following the addition of 25μM menadione. a) and b) show total and free glutathione in CHO-K1 and CHO-MRc40 respectively and c) and d) show total and free cysteine for CHO-K1 and CHO-MRc40 respectively. C, control (untreated) samples. Each bar represents the mean and standard deviation of duplicate measurements

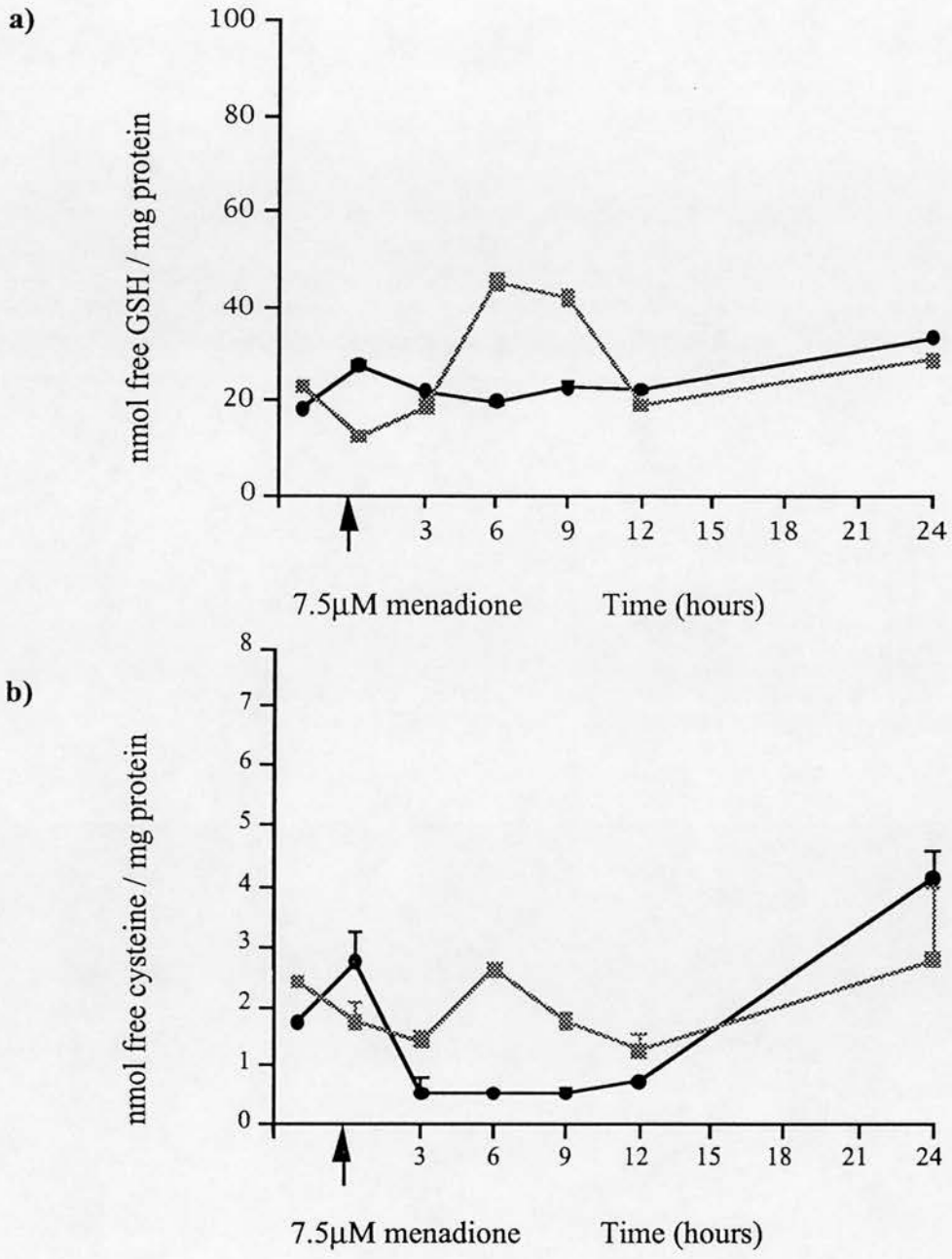


Figure 4.4 Time course of **(a)** free glutathione and **(b)** free cysteine concentration in EJ (●), and EJ-MRc30 (▩) cell lines following the addition of 7.5μM menadione to culture medium. Each data point represents the mean and standard deviation of duplicate measurements. Where not shown, error bars fall within the area of the plot symbols.

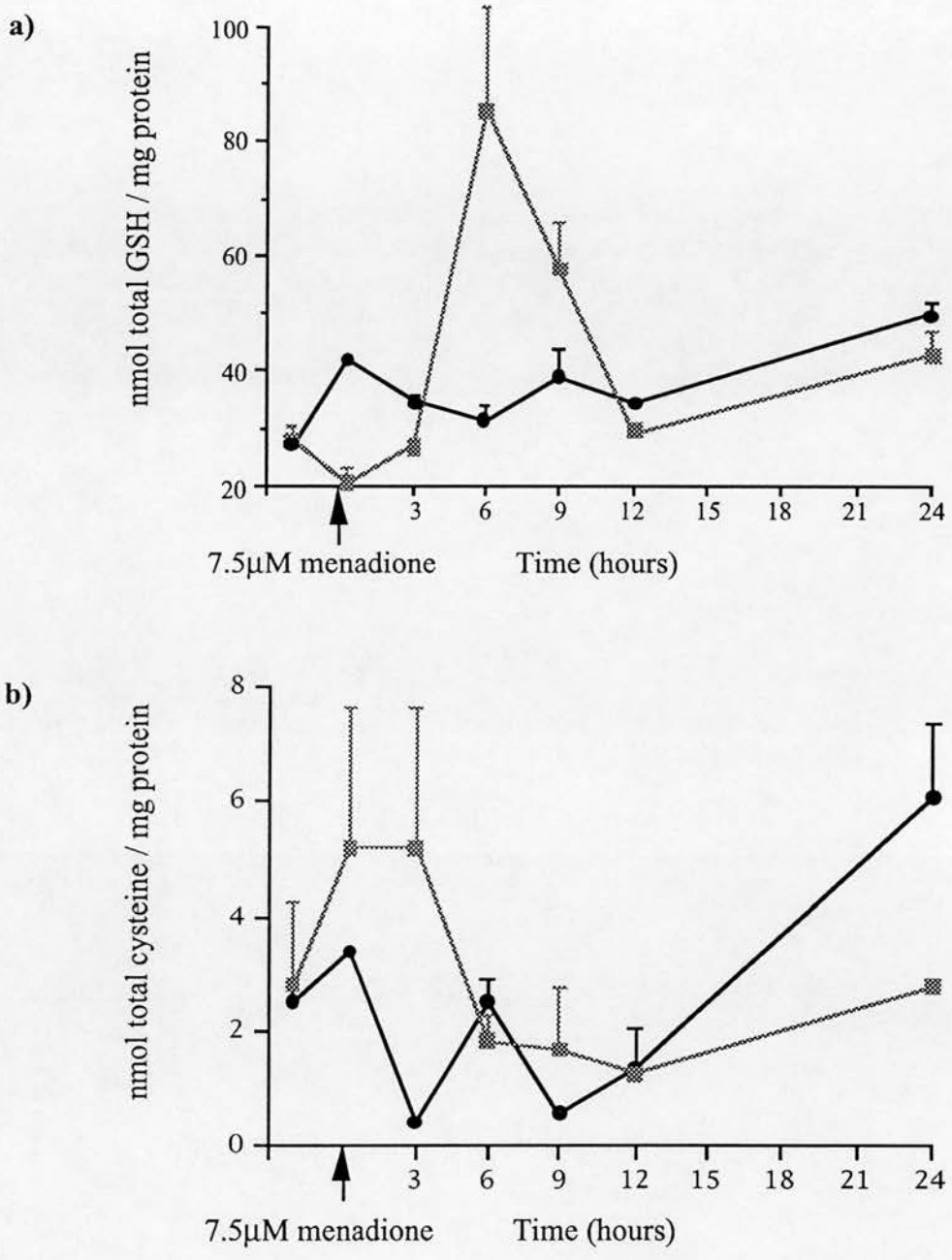


Figure 4.5 Time course of **(a)** total glutathione and **(b)** total cysteine concentration in EJ (●), and EJ-MRc30 (▩) cell lines following the addition of 7.5µM menadione to culture medium. Each data point represents the mean and standard deviation of duplicate measurements. Where not shown, error bars fall within the area of the plot symbols.

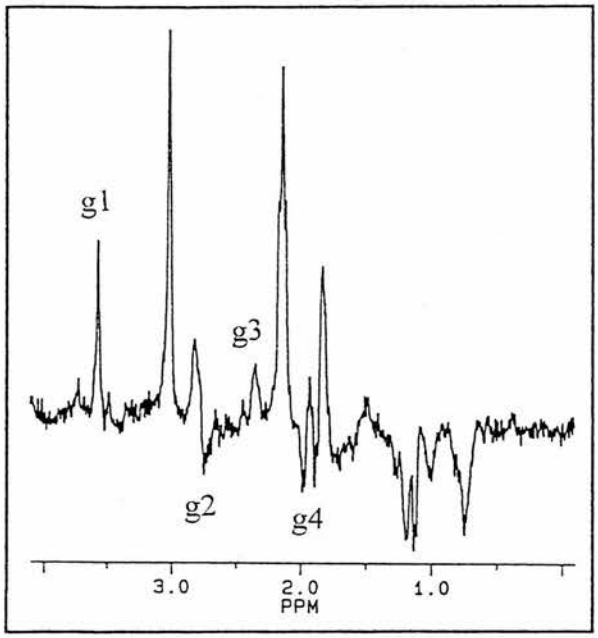
4.3.2 ^{1}H spin echo NMR spectroscopy

The ^{1}H spin echo NMR spectra of CHO-K1 and CHO-MRc40 cells are shown in Figure 4.6. Although there were more cells in the CHO-K1 sample than the CHO-MRc40 sample, the spectral patterns obtained for the two cell lines are very similar and the resonances due to glutathione can be identified in both. The amount of NMR-visible glutathione is similar in the two samples. NMR spectroscopy can be used to assess oxidation-reduction status (Livesey *et al*, 1989) and in both cell types approximately 95% of the glutathione appears to be in the reduced state. The addition of menadione to the NMR tube is followed by a number of changes over time (Figure 4.7). Two control spectra were obtained at an interval of one hour to show that the system is stable and that there is no significant change in the spectra until drug is added. This was an early experiment and menadione rather than menadione bisulphite was added to the NMR tube. Menadione is very insoluble in aqueous solution and so was added as a slurry. Two 175ml cell culture flasks were harvested to give the 10^{7} cells required for this experiment. The amount of drug added to the NMR tube was that amount which had it been added to the culture medium would have given a concentration of 25μM. The final drug concentration in the NMR tube is greater than this since it was added to a volume of only 0.5ml but the absolute amount of drug per cell would have been approximately the same as in the HPLC experiment. In Figure 4.7 it can be seen that following the addition of menadione, the peaks assigned to glutathione diminish and this is apparent from 30 minutes onwards. At 12 hours the resonances due to glutathione are barely detectable. The resonance due to lipid head groups in the cell membrane increases in intensity during the course of the experiment. These NMR experiments necessitated maintaining cells in PBS at room temperature overnight. A control experiment showed that the spectra obtained at the start and at the end of such experiments were not significantly different. Cells maintain their viability in these circumstances and so any change observed in the spectra following addition of drug can be assumed to be an effect of menadione and not due to the severe nature of the suspending medium.

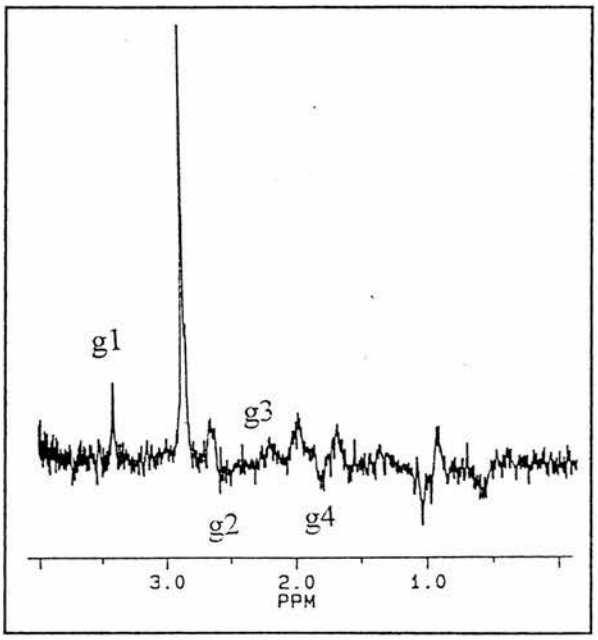
In the experiment shown in Figure 4.8, CHO-K1 cells were incubated with 100μM menadione bisulphite which caused rapid depletion of glutathione. In contrast, incubation of CHO-MRc40 cells with 100μM and then 500μM menadione bisulphite did not cause a change in intracellular glutathione. NMR spectroscopy is sufficiently sensitive to allow observation of menadione bisulphite in the cell suspensions. In both CHO-K1 and CHO-MRc40 we observed a loss of menadione from the cells (Figure 4.9) which was more rapid in CHO-MRc40 than in CHO-K1,

suggesting more efficient metabolism of menadione in the resistant cells. It is significant that in the resistant cells menadione is still being metabolised without any change in the NMR-visible pool of glutathione. In none of the NMR spectroscopy data is there any evidence to support the formation of a glutathione-menadione conjugate or substantial amounts of di-glutathione.

Figure 4.6 NMR spectra of a) CHO-K1 and b) CHO-MRc40 cells. The four resonances assigned to glutathione are indicated, g1 - g4.



a)



b)

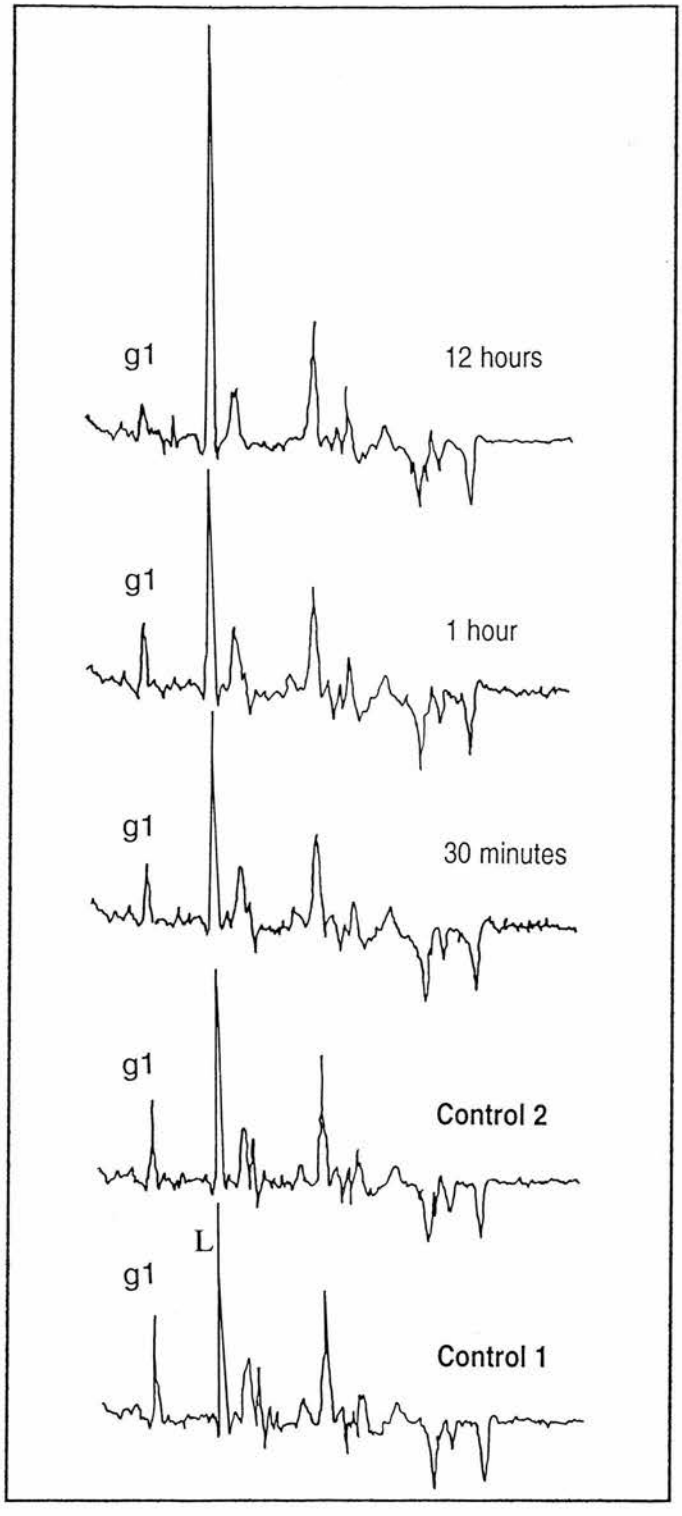
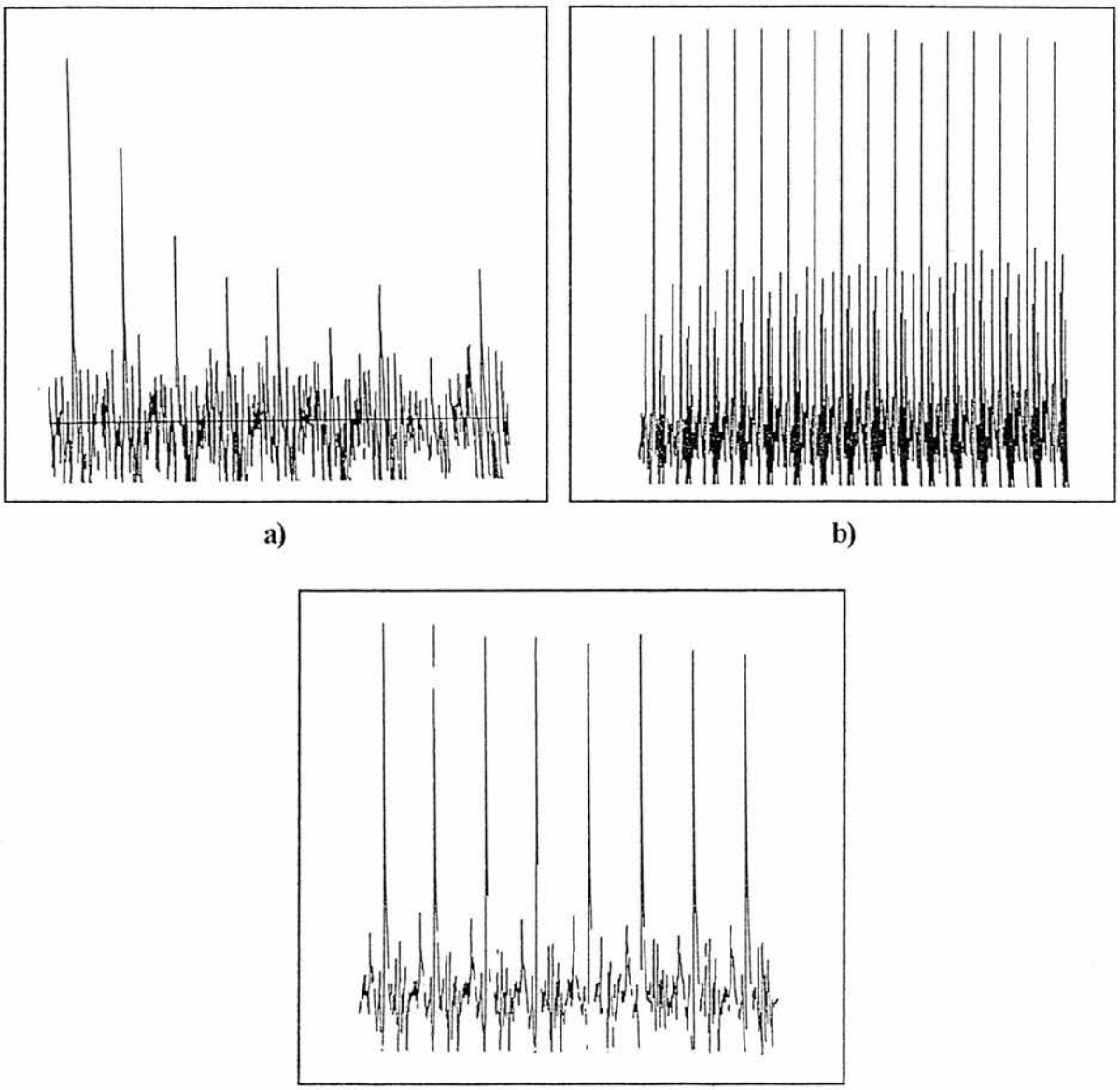


Figure 4.7 Spectra obtained at 0.5, 1 and 12 hours after the addition of 25μM menadione to CHO-K1 cells. Control spectra were taken one hour apart. L, resonance attributed to lipid head group

Figure 4.8 G1 peaks from successive spectra (obtained 30 min apart) are plotted against time for a) CHO-K1 cells following 100μM menadione bisulfite b) CHO-MRc40 cells following 100μM menadione bisulfite and c) CHO-MRc40 cells following 500μM menadione bisulfite.

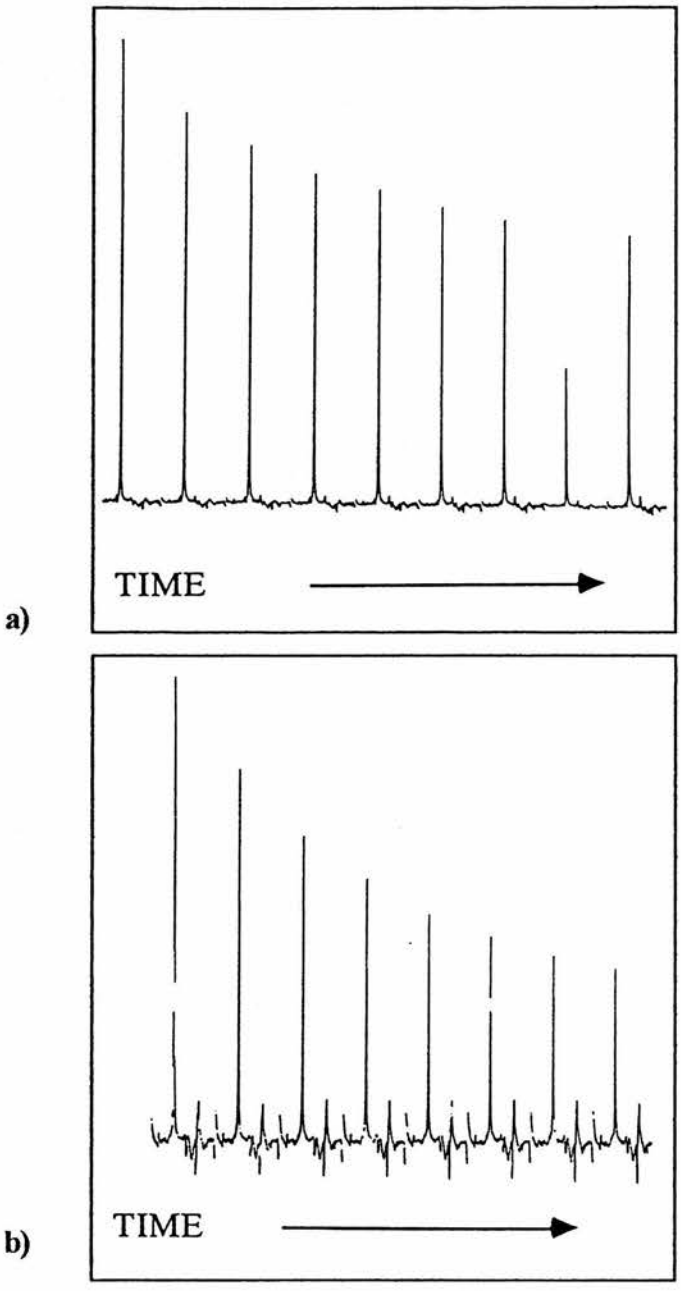


a)

b)

c)

Figure 4.9 Peak attributable to menadione bisulfite plotted against time for a) CHO-K1 and b) CHO-MRc40 cells.



4.4 DISCUSSION

Cell lines that are resistant to menadione have been isolated from parental Chinese hamster ovary and EJ cell lines. Using HPLC we found that there was a modest elevation of glutathione and cysteine in the resistant compared with the parental CHO cells. There was no significant difference in total thiol concentrations in drug-resistant and sensitive EJ cell lines although in EJ-MRc30 a greater proportion of thiols were in the reduced state compared with the drug-sensitive cells. Two other groups have measured the glutathione content in menadione-resistant cell lines which they have isolated. Martins and Meneghini (1990) found a 1.5-fold increase in glutathione in a menadione-resistant variant of V79 Chinese hamster cells. Ngo and Nutter (1994) cloned a menadione-resistant subline of the human breast carcinoma line, MCF-7. These cells showed no alteration in glutathione content although it was noted that the activity of the glutathione-related enzymes, glutathione S-transferase Pi (GST-Pi) and γ-glutamyl transpeptidase (γ-GT), was increased in the resistant cell line. Ngo postulates that the glutathione concentration is unchanged because increased consumption of glutathione (by GST-Pi for menadione-glutathione conjugate formation) is balanced by increased transport of the constituents of glutathione into the cell (one of the functions of γ-GT). The concentration of cysteine was not measured in either of these menadione-resistant cell lines. Neither was the ability of the cells to synthesise glutathione in the face of oxidative stress assessed.

Although menadione-resistant cells show only a modest increase in resting thiol concentrations compared with their parental cell lines - they appear to have a greater capacity for resynthesis of glutathione than do wild-type cells. Glutathione synthesis occurs as a result of the sequential action of the two enzymes γ-glutamyl cysteine synthetase (γ-GCS) and glutathione synthetase. γ-GCS is rate-limiting. A negative feedback mechanism operates in which glutathione inhibits γ-GCS and so inhibits its own synthesis (Richman and Meister, 1987). It seems likely that during the initial fall in thiol level after addition of menadione, feedback inhibition is released and glutathione synthesis proceeds. Others investigators have found that the cellular regulation of glutathione is more important in protection from various oxidative insults than is its overall concentration. For example, Moore *et al* (1989) found that a strain of *E. coli* enriched in the genes for γ-GCS and glutathione synthetase was more resistant to cell killing by γ-irradiation than the corresponding wild strain. In bacteria the concentration of glutathione varies markedly with the phase of growth. It is high during the stationary phase and low during the logarithmic growth. It was found that percent survival after irradiation was the same whichever the phase of growth of wild

strain bacteria when they were irradiated. It seemed that the relative radioresistance of the gene-enriched strain was associated with an increased capacity to synthesise glutathione immediately after irradiation rather than the absolute concentration of glutathione *per se*. An increased capacity for glutathione synthesis is also seen in rat hepatocytes pretreated with phenobarbital (Utley and Mehendale, 1989). Hepatocytes isolated from phenobarbital-pretreated and naive Sprague-Dawley rats were preincubated with or without 80μM BCNU and then exposed to varying concentrations of menadione. BCNU is a potent inhibitor of glutathione reductase. After the addition of menadione, the concentration of glutathione recovered to near control levels in phenobarbital-pretreated but not naive animals. This recovery occurred both in the presence and absence of glutathione reductase and is therefore likely to be the result of an increase in *de novo* synthesis of glutathione rather than to replacement of GSH through the reduction of GSSG.

The metabolism of menadione has been noted to be accompanied by rapid depletion of GSH and accumulation of GSSG in several experimental systems. In the work of Di Monte *et al* (1984a), for example, it was noted that the depletion of GSH and elevation of GSSG levels occurred within minutes of the addition of menadione to hepatocytes. In the experiment shown in Figure 4.3, however, no such rise in GSSG is seen. The preparation of samples for HPLC involves trypsinisation of cells and this procedure is well known to alter thiol redox balance (Morrison *et al*, 1985a). This may explain why there is an apparent preponderance of the reduced form of GSH even following treatment with menadione. Alternatively, it is possible that GSSG is formed but converted so rapidly back to GSH that the elevation in GSSG goes undetected as the first sample was collected at 20 minutes after the addition of menadione. In the experiments described by Di Monte the concentration of GSSG reached a peak at 5 minutes and had returned to normal by half an hour. Although we did not see a rise in GSSG following menadione treatment it is interesting that the HPLC and NMR spectroscopy data from the present study appear to support one another. In neither was there any evidence to support the formation of substantial amounts of di-glutathione.

It has been shown that glutathione synthesis is stimulated by cysteine which is one of the three constituent amino acids of glutathione (Jackson, 1969; Richman and Meister, 1987). We have demonstrated that after addition of menadione, cysteine concentration falls initially but then rises above normal levels and this is assumed to be the result of increased transport of cysteine from the medium into the cell. This response is more marked in the CHO-MRc40 and in EJ-MRc30 than in their respective parental cell lines (Figure 4.1 b and 4.4 b)). It is tempting to speculate that

one of the mechanisms by which the CHO-MRc40 line has acquired resistance to menadione is through improved efficiency of cystine uptake.

When we used NMR spectroscopy of whole cells rather than HPLC to observe glutathione metabolism we saw a rather different pattern to that in the HPLC experiments. Although the NMR-visible glutathione falls in CHO-K1 cells following the addition of menadione there is no apparent subsequent recovery. This is not surprising because during these experiments cells are suspended in PBS whereas for the HPLC experiments cells are growing in media containing cystine. The opportunity to resynthesise glutathione would therefore be limited. Brown *et al* (1977) carried out similar experiments, using NMR spectroscopy to follow the response of erythrocytes to treatment with the oxidising agent, terbutyl hydroperoxide. Terbutyl hydroperoxide was added to sugar-depleted cells. A profound depletion of reduced glutathione was noted and, because the cells were metabolically dormant, no recovery of glutathione was observed. However, as soon as glucose was added, the NMR resonances for reduced glutathione were again seen. Although we did not see a recovery of glutathione in either CHO-K1 or CHO-MRc40 cells, the menadione-resistant cells were better able to maintain the pool of intracellular glutathione even in the face of considerable oxidative stress. It is possible that the resistant cells are richer in reductants such as NADPH which would be capable of maintaining the redox status.

There are several reports in the literature of the identification, usually by electron spin resonance spectroscopy, of semiquinone and hydroquinone radical formation during the intracellular metabolism of menadione (Miura *et al*, 1992). There is little evidence in the NMR spectra presented here to support the efficient formation of either a menadione hydroquinone or semiquinone via mitochondrial oxidation. If a hydroquinone or semiquinone was produced it would not itself be NMR-visible. However, the associated paramagnetism would be expected to significantly broaden the resonances in the spectra and this is not observed. The generation of low concentration radicals is observed to affect T2, altering the shape of the FID and the strength of the lock signal. Neither is observed here. Although we were unable to demonstrate the formation of a semiquinone in this system this may be because this process is dependent on pH and the relative concentrations of menadione and glutathione. Using ESR for detection, Miura *et al* found that formation of the semiquinone radical was maximal at a pH of 9.0 and lowest at pH 7.0 (Miura, 1992). The cells in our NMR experiments were suspended in PBS (pH 7.4) so semiquinone formation would be expected to be low.

We have demonstrated marked alteration in the metabolism of glutathione in

menadione-resistant compared with sensitive cells and it is likely that this alteration contributes to the resistance phenotype. Further evidence to support this hypothesis is that exogenous glutathione had a protective effect against menadione growth inhibition in mouse L1210 leukaemia cells (Akman *et al*, 1985) and in human KB cells (Nutter *et al*, 1991). The intacellular glutathione content varies with the phase of cell growth, being maximal during stationary phase. Certainly it was observed that the cellular response to menadione varied with cell density. When cells were in stationary phase they were more tolerant of menadione. Nutter *et al* noticed the same phenomenon and report that the LD_{50} of menadione bisulphite in KB cells increased with increasing cell density. When cells were plated at a density of 5×10^5 and after 24 hours treatment with menadione for 1 hour, the LD_{50} was 100μM. However, the LD_{50} was 30μM in cells that had been plated at a lower initial density (5×10^4).

4.5 SUMMARY

It is clear from HPLC and NMR studies that the metabolism of glutathione is altered in menadione-resistant compared with drug-sensitive cell lines. There are modest increases in the resting levels of both GSH and cysteine in menadione-resistant CHO cells compared with the parental line from which they were isolated. Perhaps more importantly the difference in thiol concentrations between the two cell lines is greatest during the recovery phase after an oxidative insult. This work indicates that the concentration of low molecular weight thiols is an important aspect, but may not be the only factor, in determining menadione resistance.

CHAPTER FIVE

GLUTATHIONE-RELATED ENZYMES AND STRESS-INDUCIBLE PROTEINS IN MENADIONE RESISTANCE

5.1 INTRODUCTION

The observation that glutathione metabolism was altered in menadione-resistant cell lines prompted a study of the expression of glutathione-dependent enzymes in these cells. Glutathione-dependent enzymes are amongst those that protect cells against free radical damage and are implicated in cellular resistance to cytotoxic drugs. Glutathione is thought to play a role in the regulation of the oxidative-stress inducible enzyme, heme oxygenase, and the expression of heme oxygenase was therefore also studied. The expression of several other enzymes that have been implicated in protection from oxidative stress including the metallothioneins, catalase, superoxide dismutase and QAO were also investigated. The CHO cells have a shorter doubling time than EJ and so were, in general, easier to work with. For this reason most of the work described below was carried out on CHO-K1 and CHO-MRc40. A few experiments were repeated in EJ cells.

5.1.1 The glutathione-dependent enzymes

(a) Glutathione S-transferases

There are several reasons to suppose that GST expression might be altered in menadione-resistant cells. Firstly, the overexpression of GST-Pi has frequently been observed in multidrug-resistant cell lines (Hayes and Wolf, 1988). The functional role of GST-Pi overexpression in drug resistance has long been debated (Campling *et al,* 1993; Yusa *et al*, 1988). However, since it is so often observed and since there is often co-ordinate expression with other drug resistance genes it seems probable that the GST enzymes do play an active role in the drug resistance phenotype. For example, a potential role for GST-Pi is in the detoxification of the free radical-induced damage caused by quinones such as adriamycin (Batist *et al*, 1986).

Secondly, detoxification of quinones themselves may occur via a GST pathway (Morgenstern *et al.* 1981). Thirdly, it has been postulated that GSTs play a role in removal of the toxic products of lipid peroxidation. It is known that the normal cellular metabolism of lipid peroxides produces 4-hydroxyalkenals such as 4-hydroxynonenal and 4-hydroxydecanal (Poot *et al*, 1988). These molecules together with fatty acid hydroperoxides (such as those of linoleic acid) have been shown to be effective substrates for GST isozymes (Ketterer, 1986). Lastly, GST isozymes, although they do not show activity towards hydrogen peroxide (Mannervik *et al*, 1985) do so towards the organic peroxides that may be formed during menadione-induced oxidative stress.

The other classes of GST isoenzyme have also been implicated in drug resistance. Overexpression of alpha and mu class GST has been reported in Walker 256 rat mammary carcinoma cell lines with acquired resistance to chlorambucil (Wang and Tew, 1985; Buller *et al*, 1987). In addition, alpha and pi class are elevated 50- and 2-fold respectively in a Chinese hamster ovary cell line resistant to bi-functional alkylating mustards (Lewis *et al*, 1988b). Alpha class GST has selenium-independent glutathione peroxidase activity and so the expression of this enzyme in menadione-resistant cells was of interest.

(b) Glutathione peroxidase

Two major types of GSHPx have been identified. A selenoenzyme containing selenium in the form of covalently bound selenocysteine at its active site and a non-selenium containing protein with negligible activity towards hydrogen peroxide. As discussed above the latter activity has been associated with the glutathione S-transferase family of enzymes (Prohaska and Ganther, 1977).

The expression of GSHPx in menadione-resistant cells was of interest because this enzyme has been reported to be elevated in a number of drug-resistant cell lines and protects cells from oxidative damage. In particular, glutathione peroxidase catalyses the reduction of lipid hydroperoxides and of peroxidised DNA. Although most cellular GSHPx activity is located in the cytosol, low levels are also present in the mitochondria. Mitochondrial GSHPx is involved in the regulation of mitochondrial substrate oxidations and in the protection of mitochondrial enzymes and DNA from lipid peroxidative damage.

Several studies have shown that GSHPx contributes to drug resistance in some cases. Sinha *et al* (1989) showed that a human adriamycin-resistant cell line (MCF-7 ADR^R) had a 12- to 14-fold elevation in GSHPx activity compared with the parental

line from which it was isolated. The adriamycin-resistant cells were 5-fold more resistant to H_2O_2 than the wild type and this effect was attributed to the increased levels of GSHPx in the drug-resistant line. The role of GSHPx was assessed directly by adding either purified enzyme or ADR^R cell lysate, containing elevated GSHPx activity, to the WT cells. Both of these inhibited the formation of ·OH in the WT cells (Sinha *et al*, 1989). Adriamycin resistance acquired *in vivo* in several tumour cell lines was found to be associated with increased levels of glutathione peroxidase (Cowan *et al*, 1986).

Mirault *et al* (1991) used a different strategy to study the role of GSHPx in the protection of cells from oxidative damage. T47D human breast cells, which are known to have a low constitutive level of GSHPx activity, were transfected with expression vectors designed to overexpress human GSHPx efficiently. Stable transfectants that overexpressed the enzyme by 10 to 100 times more than the parental cells were isolated and characterised. Compared to parental cells T47D transfectants expressing high levels of GSHPx were more resistant to transient exposure to cytotoxic concentrations of H_2O_2, cumene hydroperoxide or menadione as judged by growth inhibition kinetics, or DNA strand breakage. Extensive DNA breakage induced by menadione was markedly reduced in cells overexpressing GSHPx. DNA from cells exposed to cytotoxic concentrations of menadione for one hour was extracted 2 hours post-exposure and 3'-end-labelled with α-^{32}PdCTP. The radioactivity incorporated into DNA was found to be linearly related to the number of DNA strand breaks. Results showed that DNA breakage was 6 to 9 times less extensive in the GSHPx overexpressing cell lines compared with controls.

(c) γ-Glutamylcysteine synthetase

γ-GCS catalyses the first and rate-limiting step of glutathione biosynthesis. The reaction is feedback inhibited by glutathione in a competitive fashion. The amino acid sequence of rat kidney γ-glutamylcysteine synthetase was published in 1989 (Yan and Meister, 1990) and since then a full-length cDNA clone for the human liver γ-glutamylcysteine synthetase has been sequenced (Gipp *et al*, 1992). The availability of a full-length cDNA clone for human γ-GCS has permitted the investigation of the regulation of this enzyme. There have been a small number of reports of the up-regulation of γ-glutamylcysteine synthetase activity. For example, γ-glutamylcysteine synthetase activity was increased in a melphalan-resistant human multiple-myeloma cell line expressing increased glutathione levels (Mulcahy *et al*, 1994). γ-glutamylcysteine synthetase activity and mRNA was also elevated in

quinone-treated rat lung epithelial cells (Shi *et al*, 1994).

(d) γ-Glutamyltranspeptidase

This enzyme is responsible for the cleavage of the unique γ-glutamylcysteine peptide bond of glutathione. It is a membrane-bound enzyme with its active site directed toward the outside of the cell. Cysteinylglycine, a product of the γ-GT catalysed reaction, is metabolised by a dipeptidase - and the free amino acids are transported inside the cells (Horiuchi *et al*, 1978). Elevation of γ-GT has frequently been observed in cell lines selected for drug resistance. For example, a Chinese hamster ovary cell line selected for chlorambucil resistance had a 3.5-fold increase in γ-GT activity (Lewis *et al*, 1988b). PE01 and PE04 are cell lines derived from a patient with ovarian adenocarcinoma before (PE01) and after (PE04) the onset of clinical chemoresistance (Wolf *et al*, 1987). The level of γ-GT was elevated 6-fold in PE04 (Lewis *et al*, 1988c).

There is some evidence that the action of γ-GT attenuates quinone-induced cellular damage. In a report by Stenius and Högberg (1988) γ-GT was found to protect cells from the deleterious effects of the quinone compound, hydroquinone. Hepatocytes were isolated from carcinogen treated rats that had received phenobarbital for three weeks. Isolated cells were then incubated in buffer containing hydroquinone which depletes glutathione. Immunocytochemistry was used to assess the γ-GT-content of the cells that survived this treatment. It was found that the toxicity related to glutathione depletion increased the proportion of γ-GT-positive hepatocytes from 10 to 15% up to 40 to 60% indicating that the toxicity mainly affected γ-GT-negative cells.

(e) Glutathione reductase

Glutathione reductase (GR) is a flavoprotein which catalyses the NADPH-dependent reduction of glutathione disulphide to glutathione. GR is found in almost all tissues and cell types and functions to maintain glutathione in the reduced state. The enzyme has been shown to be induced by compounds such as TPA and metals such as selenium (Chung and Maines, 1981). BCNU is known to inhibit the function of glutathione reductase (Tew *et al*, 1985). The activity of glutathione reductase is likely to be limited by high concentrations of menadione because of the associated depletion of reduced pyridine nucleotides. Glutathione reductase has only rarely been reported to be elevated in drug-resistant cell lines.

5.1.2 Other antioxidant enzymes

(a) Heme oxygenase

Heme oxygenase is strongly induced by oxidative stress in mammalian cells and it is thought to contribute to the cellular antioxidant defence system. Heme oxygenase expression has been studied in two drug-resistant cell lines to date. The findings were contradictory. The basal activity of heme oxygenase in two menadione-resistant MCF-7 cell lines was significantly less than that of the parental MCF-7 cell line from which they were derived. This suggested that heme oxygenase had no functional role in menadione-resistance (Nutter *et al*, 1994). However, in an arsenite-resistant cell line that showed cross-resistance to menadione, heme oxygenase activity was elevated. When this cell line was grown in drug-free medium, arsenite-resistance was found to decline in parallel with decreasing heme oxygenase activity (Lee and Ho, 1994). Although it was not proven that these events were causally related the implication was that heme oxygenase might contribute to the resistance phenotype.

(b) Metallothionein

The metallothioneins are a group of small, cysteine-rich proteins whose primary function is the protection of cells from the deleterious effects of heavy metals. The abundance of SH groups also means that the metallothioneins have considerable reducing power and so play a role in defence against oxidative stress. The expression of metallothioneins has been studied in a number of drug-resistant cell lines. Mello-Filho *et al* (1988) rendered V79 cells resistant to cadmium by the stepwise increase of cadmium concentration in the medium until cells were able to grow in the presence of 200μM cadmium sulphate. These cells exhibited resistance to the oxidising agents ionising radiation and H_2O_2. A similar result was obtained when mouse fibroblast cells were maintained in cadmium (100μM) in culture. The cadmium-treated cells were shown to have high metallothionein levels and they exhibited resistance to ionising radiation. After a dose of 3Gy the survival levels of the parental mouse fibroblast line and its metallothionein-rich derivative were 39% and 74% respectively (Bakka *et al*, 1982). However, elevated metallothionein activity is not always associated with resistance to reactive oxygen species. The Chinese hamster ovary cell line, CHO-K1 and an X-ray sensitive derivative, xrs-2, were transfected with a bovine papillomavirus-linked construct carrying the human MTIIA gene (Kaina *et al* 1990). Transfectants, which contained 25 to 166 times more metallothionein than

parent cells, exhibited a 40-fold increased survival after treatment with cadmium chloride. However, the fold resistance of individual transfected clones did not correlate directly with the amount of metallothionein they contained. Although resistant to the alkylating agents MNU (N-methyl-N-nitrosoureas) and MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), the transfectants did not show cross-resistance to bleomycin or ionising radiation. This suggested that the free radicals produced by these agents were not scavenged efficiently by metallothioneins *in vitro*. Interestingly, when the metallothionein-transfected cells were treated with buthionine sulphoximine there was increased sensitivity to ionising radiation. The authors of this report interpreted this as evidence that metallothioneins were not able to replace glutathione and act as scavengers of free radicals.

Chan *et al* (1992) attempted to define the relative roles of glutathione and metallothionein in menadione-resistance. The model system used consisted of rat liver slices which were incubated for 3 hours with menadione at concentrations of 100 to 300μM. Cellular toxicity was assessed by elevation of intracellular calcium, depletion of glutathione and leakage of lactate dehydrogenase (LDH). Pretreatment of rats with $ZnSO_4$ led to a 30-fold elevation of metallothionein and this was associated with suppression of menadione toxicity. Even when glutathione was depleted by means of buthionine sulphoximine the metallothionein-rich liver slices showed less toxicity when treated with menadione than did the control liver samples. This suggested that metallothionein had the ability to partially compensate for a low glutathione concentration. The authors concluded that metallothionein does protect against quinone-induced toxicity but that its role is secondary to that of glutathione.

These various reports prompted a study of metallothionein expression in menadione-resistant CHO cell lines.

5.2 STRATEGIES

In order to establish whether menadione-resistance was associated with changes in the expression of enzymes known or thought to be involved in defence against oxidative stress, northern blot analysis was carried out to compare their expression in wild-type and resistant cells. It appeared that several genes associated with transient response to oxidative stress were constitutively over-expressed in menadione-resistant cell lines. Experiments were therefore designed to establish how wild-type and drug-resistant cell lines responded to menadione-induced stress. Cells were seeded into culture dishes and allowed to grow to 70% confluency Growth medium was removed and retained. Cells were rinsed with isotonic phosphate buffered saline

and menadione added at a concentration of 20μM in PBS at 37°C. Control flasks were treated with PBS. After 30 minutes the menadione was removed and the preconditioned medium replaced. Incubation was then continued until cells were harvested for RNA at 1/2, 1, 2, 4, 6, 8 and 24 hours. Northern blot analysis was carried out and the kinetics of mRNA induction in response to oxidative stress assessed.

To quantify the amount of various classes of GST isoenzymes in these cell lines, Western blots were performed using antibodies raised against alpha, mu and pi classes of GST. Western blot analysis was also used to study the expression of two other antioxidant enzymes, SOD and catalase, in menadione-resistance. The activity of putative stress-inducible proteins was measured using the assays described in section 2.11.

5.3 RESULTS

5.3.1 Constitutive expression of glutathione-dependent and other stress genes

All three menadione resistant cell lines exhibited a significant increase in the level of glutathione S-transferase Pi mRNA (Figure 5.1a). The mRNA encoding the α class GSTs were not detected in any of the cell lines. The increase in GST-Pi mRNA did not parallel the fold resistance observed. Interestingly there was only minimal elevation of GST-Pi mRNA in the EJ menadione-resistant cell lines compared with EJ-WT (Figure 5.5). A significant increase in glutathione peroxidase mRNA was also observed in the CHO cell lines resistant to 30 and 40μM menadione (Figure 5.1b). Although there were slight differences in the mRNA loading it appears that the level of glutathione peroxidase message relates to the relative level of menadione-resistance.

There was no difference in γ-glutamyl cysteine synthetase, glutathione reductase or metallothionein II mRNA levels in the CHO menadione-resistant cell lines compared with the wild-type cell line (Figure 5.2a, 5.2b and 5.3). Of the mRNA species studied, the most profound change observed was in the level of heme oxygenase (Figure 5.4). The level of mRNA encoding this protein was 6-fold higher than in controls. The increased mRNA was stable for several weeks in the absence of drug for 12 weeks (Figure 5.10). However, reduction in the heme oxygenase mRNA was observed. This is interesting in view of the finding that at this time point the cells also exhibited increased sensitivity to menadione.

5.3.2 Response to transient oxidative stress

Administration of menadione to the wild type cell line resulted in a significant increase in GST-Pi, glutathione peroxidase and heme oxygenase mRNA (Figures 5.6 a and d and 5.9 a). No change in γ-glutamylcysteine synthetase or glutathione reductase mRNA levels were observed (Figure 5.6 b and c). The change in heme oxygenase mRNA was most pronounced and peaked at four hours after menadione administration. Interestingly, the glutathione peroxidase level peaked at 1 to 2 hours following administration of this compound. mRNA encoding both of these proteins reverted to control levels after 24 hours. This was unlike the case for glutathione transferase Pi which remained elevated at this point. This is consistent with the long half life of this mRNA species (G. Moffat, personal communication). When a higher concentration of menadione (40μM) was used to induce oxidative stress in CHO-K1 the elevation of GST-Pi mRNA was more pronounced (Figure 5.7). The timing of the induction was, however, the same as when the concentration of menadione used was 20μM.

In contrast to the marked response to oxidants observed in the wild-type cell line, the menadione-resistant (MRc40) cell line did not show such dramatic changes in mRNA content. Glutathione S-transferase pi and glutathione peroxidase levels remained unchanged after menadione treatment (Figure 5.8 c and d). As in the wild-type there was no change in γ-glutamylcysteine synthetase or glutathione reductase levels (Figure 5.8 a and b). Heme oxygenase levels did approximately double following menadione administration, showing maximum increase after 4 to 6 hours (Figure 5.9b). As in the wild-type cell line the heme oxygenase mRNA had returned to its pretreatment level by 24 hours.

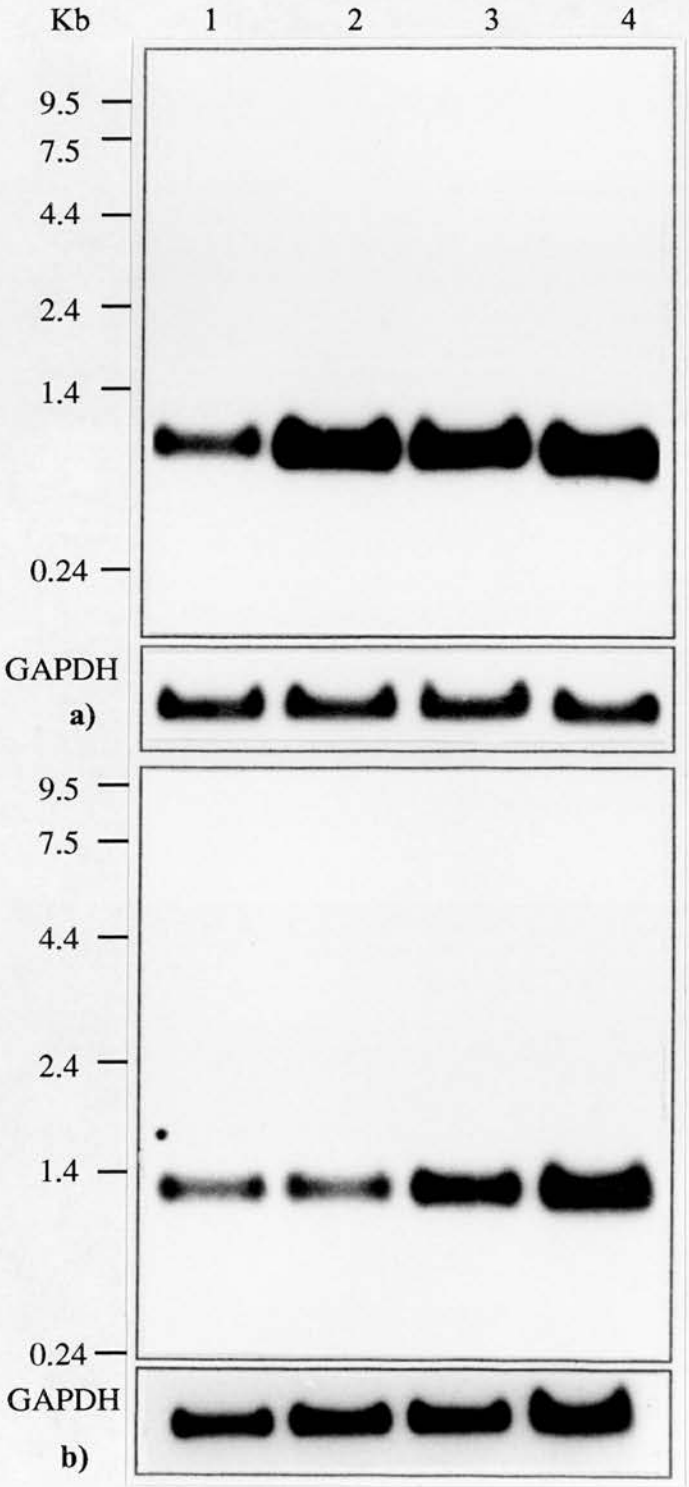


Figure 5.1 Northern blots showing expression of **(a)** GST-Pi and **(b)** GSHPx mRNA in CHO-K1 (lane 1), MRc20 (lane 2), MRc30 (lane 3) and MRc40 (lane 4). 15µg of RNA were loaded in each lane. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probes derived from the 0.8kb *Eco*R1 fragment of human GST-Pi cDNA and the 0.7kb *Eco*R1 fragment of the mouse glutathione peroxidase cDNA. Filters were reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

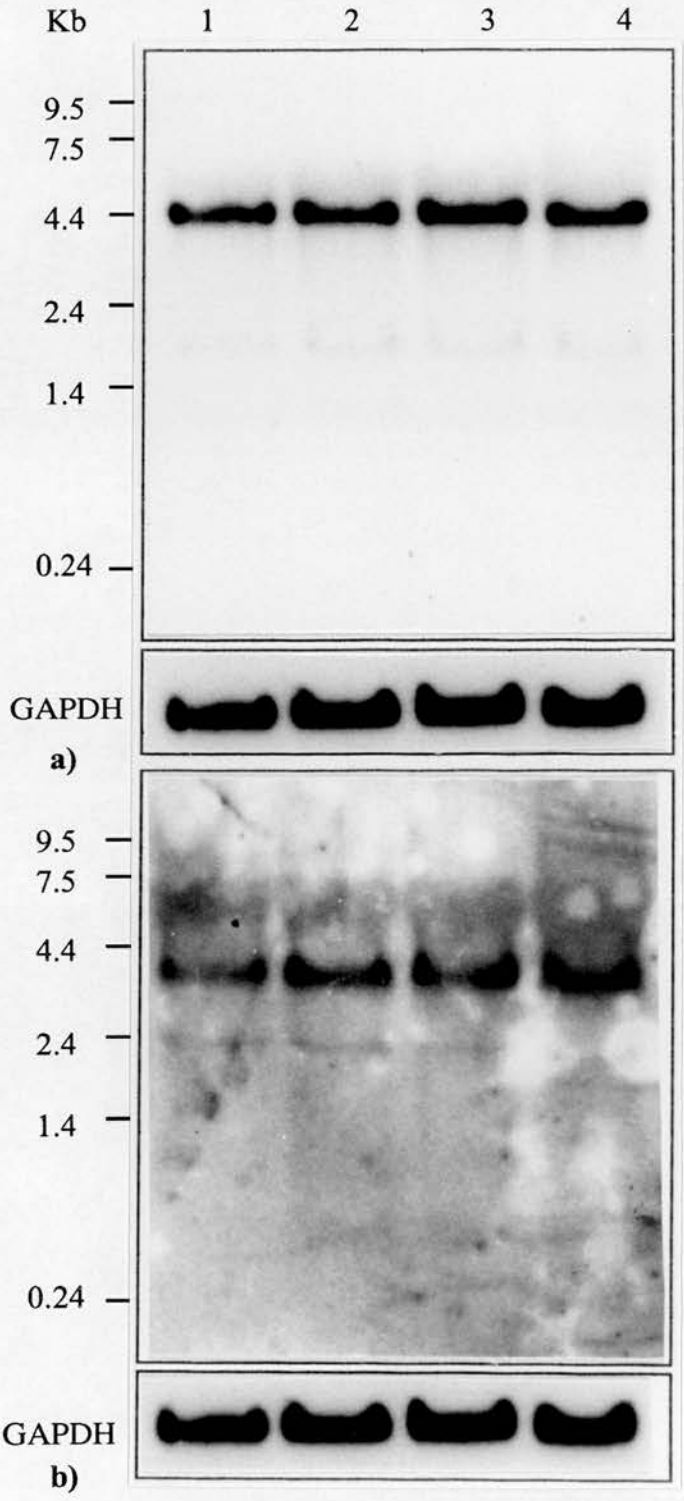


Figure 5.2 Northern blot showing expression of **(a)** γ-GCS and **(b)** GR mRNA in CHO-K1 (lane 1), MRc20 (lane 2), MRc30 (lane 3) and MRc40 (lane 4). 15μg of RNA were loaded in each lane. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probes which were obtained as described in section 2.5.5. Filters were reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

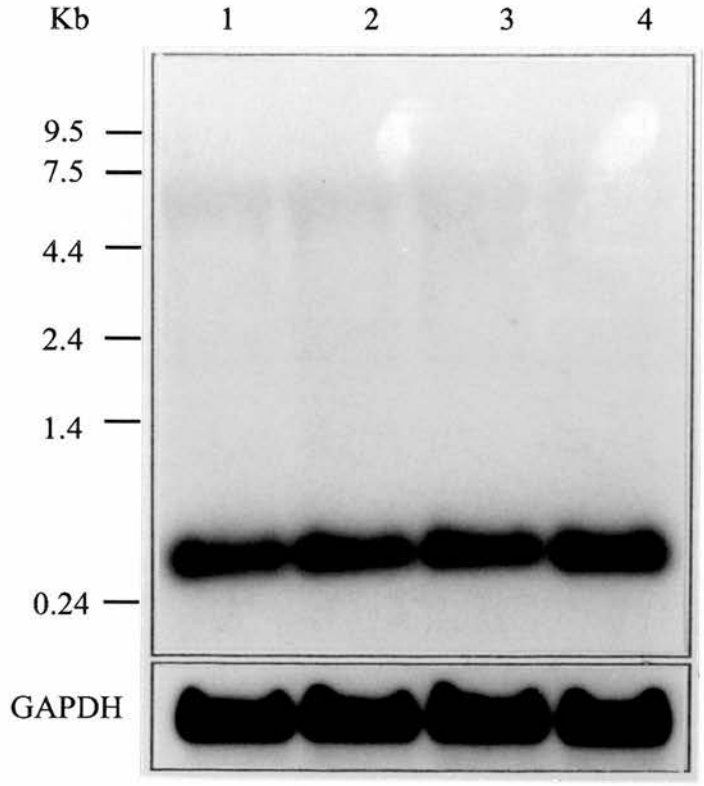


Figure 5.3 Northern blot showing expression of metallothionein in CHO-K1 (lane 1), MRc20 (lane 2), MRc30 (lane 3) and MRc40 (lane 4). 15µg of RNA were loaded in each lane. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probe. The filter was reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

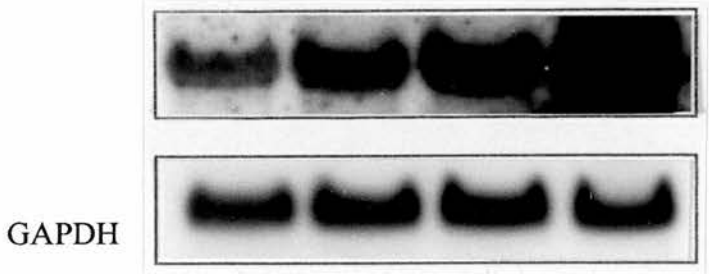


Figure 5.4 Northern blot showing expression of heme oxygenase in CHO-K1 (lane 1), CHO-MRc20 (lane 2), CHO-MRc30 (lane 3) and CHO-MRc40 (lane 4). 15µg of RNA were loaded in each track. The DNA probe for HO was derived from the 1.0kb *Eco*R1 fragment of heme oxygenase cDNA. The blot was reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

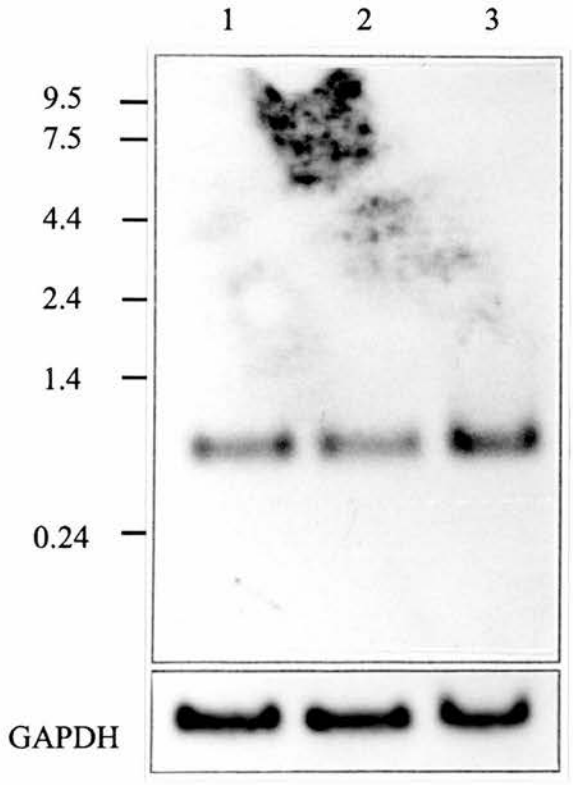


Figure 5.5 Northern blot showing expression of GST-Pi in EJ-WT (lane 1), MRc20 (lane 2) and MRc30 (lane 3). 15μg of RNA were loaded in each lane. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to a ^{32}P-labelled probe derived from the 0.8kb *Eco*R1 fragment of human GST-Pi cDNA. The filter was reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

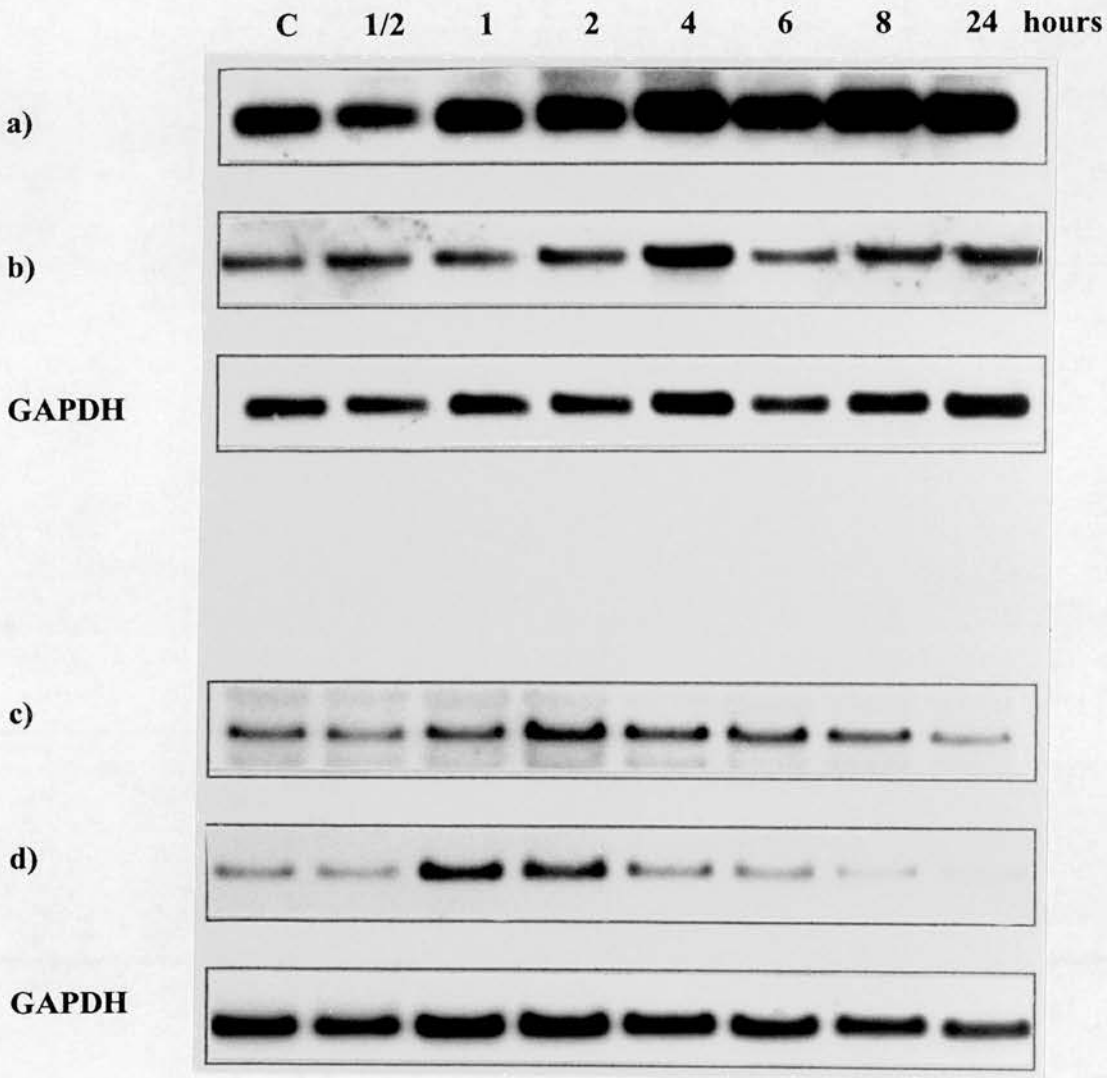


Figure 5.6 Northern blots showing the kinetics of mRNA accumulation in CHO-K1 following treatment with 20μM menadione for 30 minutes. **(a)** GST-Pi, **(b)** γ-GCS, **(c)** GR and **(d)** GSHPx. C, control (untreated) sample. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probes as described above. Filters were reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

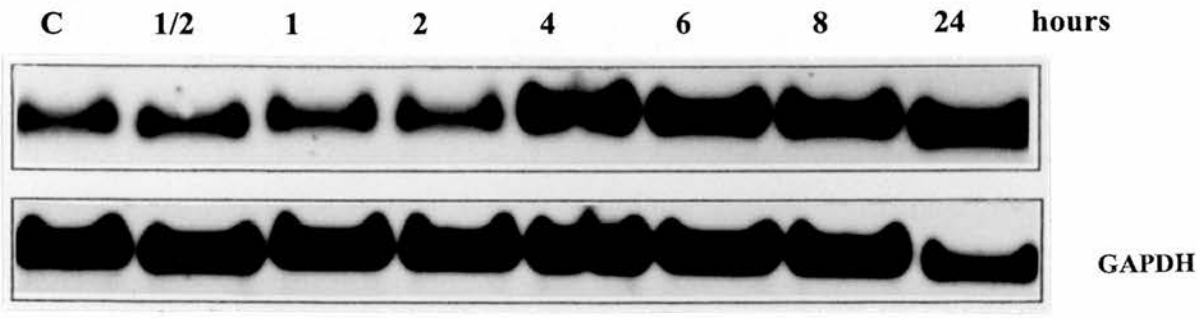


Figure 5.7 Northern blot showing the kinetics of GST-Pi mRNA accumulation in CHO-K1 following treatment with 40μM menadione for 30 minutes. C, control (untreated) sample. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probes as described above. The filter was reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

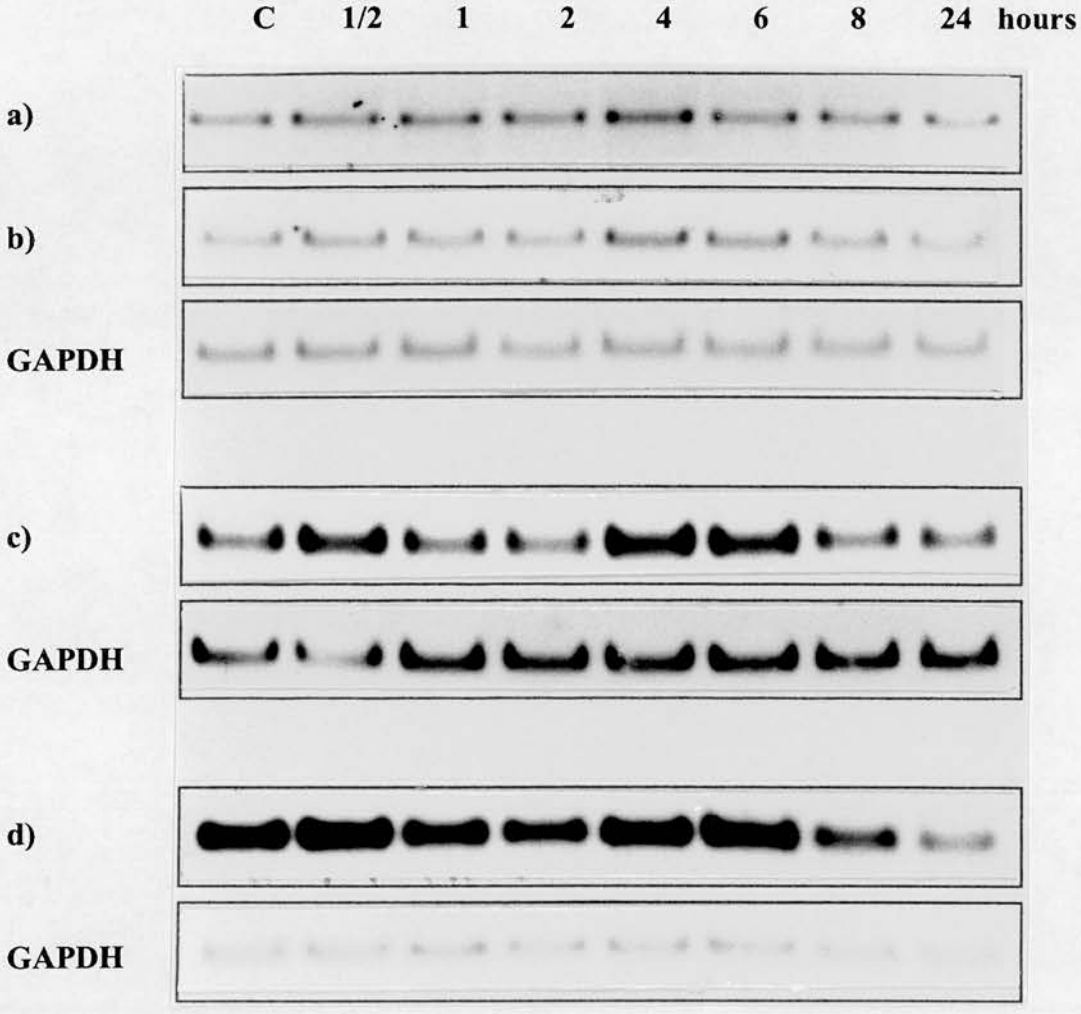


Figure 5.8 Northern blots showing the kinetics of mRNA accumulation in CHO-MRc40 following treatment with 20μM menadione for 30 minutes. **(a)** γ-GCS, **(b)** GR, **(c)** GST-Pi and **(d)** GSHPx. C, control (untreated) sample. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to ^{32}P-labelled probes as described above. The filters were reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

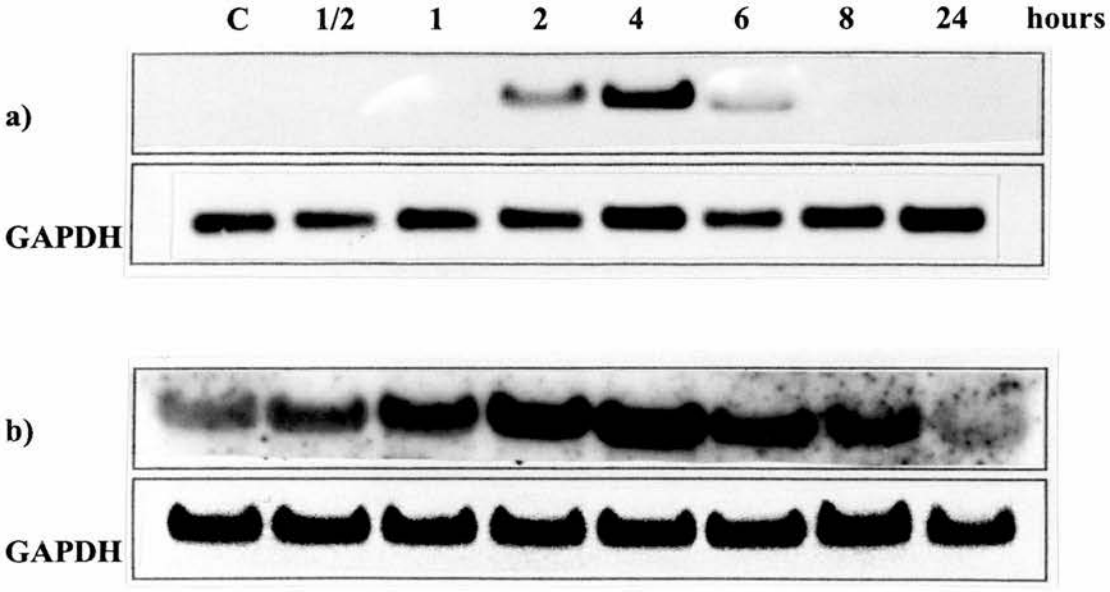


Figure 5.9 Northern blots showing the kinetics of heme oxygenase mRNA accumulation in **(a)** CHO-K1 and **(b)** CHO-MRc40 following treatment with 20μM menadione for 30 minutes. C, control (untreated) sample. After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to a ^{32}P-labelled probe derived from the 1.0kb *Eco*R1 fragment of heme oxygenase cDNA. Filters were reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

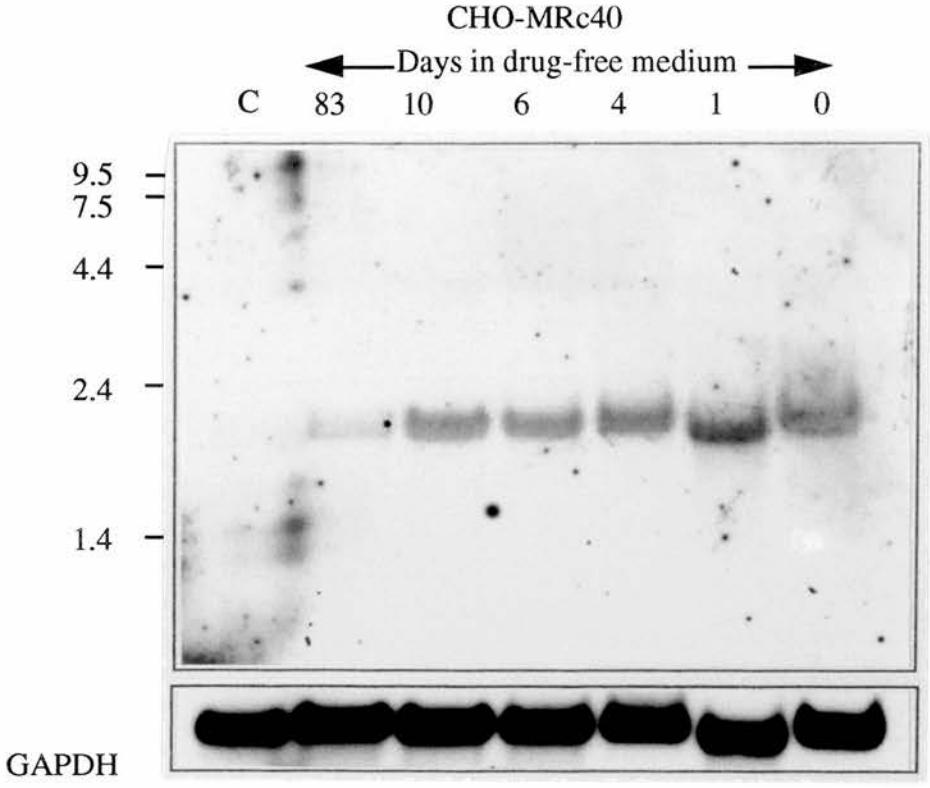


Figure 5.10 Northern blot showing HO mRNA expression in CHO-K1 (C) (lane 1) and CHO-MRc40 grown in 40μM menadione (lane 7) and in drug-free medium for the indicated lengths of time (lanes 2 - 6). After MOPS/formaldehyde electrophoresis, RNA was transferred to nitrocellulose membrane and hybridised to a ^{32}P-labelled probe derived from the 1.0kb *Eco*R1 fragment of heme oxygenase cDNA. The filter was reprobed using the *Pst*1 fragment of the glyceraldehyde phosphate dehydrogenase gene as a loading control.

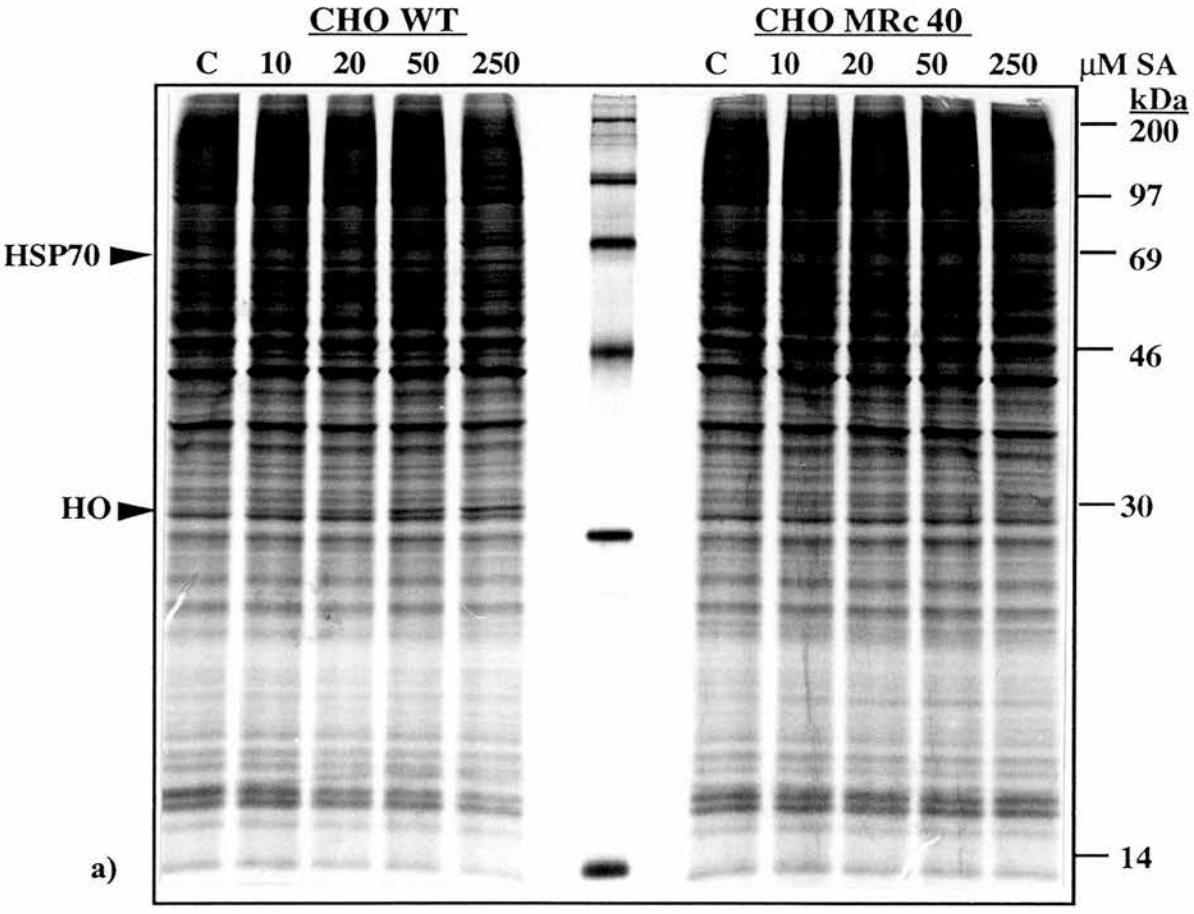
5.3.3 Induction of HO protein

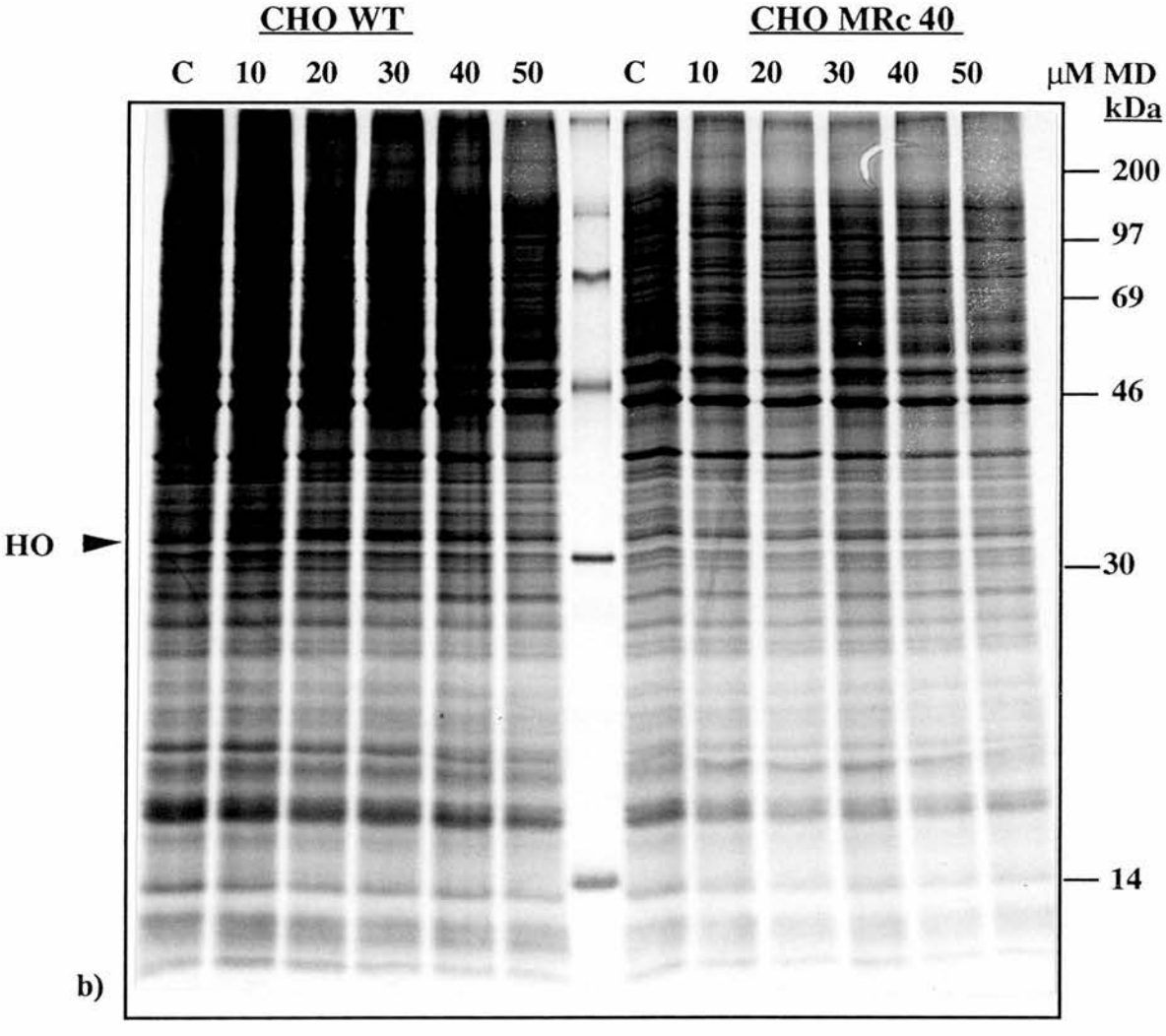
The sulphydryl-reactive agent, sodium arsenite, is one of the most powerful inducers of HO. When menadione-sensitive cells were treated with sodium arsenite and then whole cell protein analysed, the induction of HO was clearly visible (Figure 5.11a). However, HO does not accumulate to such an extent in resistant cells following the same treatment. Similarly, HO accumulation is seen in the sensitive cell line but not in the resistant cell line after treatment with menadione (Figure 5.11b). The higher concentrations of menadione used in this experiment (40μM and 50μM) are sufficient to cause cell necrosis in the sensitive cell line and this may account for the relative paucity of HO in CHO-K1 after treatment at these concentrations. It is of interest that induction of a 70-kDa protein (presumed to be HSP70) is seen in sensitive but not resistant cells after sodium arsenite.

5.3.4 Western blot analysis

Western blot analysis using antibodies against Pi, mu and alpha GST were performed to assess further the role of GST isozymes in menadione-resistance (Figure 5.12). There was only a modest (2-fold) increase in the expression of GST-Pi and no change in expression of alpha GST. Mu GST was undetectable in both the drug-sensitive and -resistant cell lines. All three menadione-resistant cell lines showed the same degree of induction of GST-Pi which was therefore not apparently related to the concentration of menadione used to select the cell lines. Interestingly when a similar experiment was performed using EJ cell lines, GST-Pi was again induced and the degree of induction now seemed to reflect the degree of resistance of the cells to menadione (Figure 5.13). The possible role of the two antioxidant enzymes, catalase and superoxide dismutase was also assessed by Western blot analysis. Figure 5.14 shows that there was no alteration in either of these proteins compared with wild-type cells.

Figure 5.11 (See page 151 and 152) Induction of heme oxygenase by **(a)** sodium arsenite and **(b)** menadione in CHO-K1 and CHO-MRc40 cell lines. Following incubation at 37°C for 2 hours, cells were radiolabelled with ^{35}S-methionine for 1 hour, proteins were then analysed by one-dimensional SDS-PAGE and autoradiography. Molecular mass markers are indicated on the right and the position of HO and HSP70 on the left.


CHO WT
CHO MRc 40
C
10
20
50
250
C
10
20
50
250
μM SA
kDa
200
97
69
46
30
14
HSP70
HO
a)


CHO WT
CHO MRc 40
C 10 20 30 40 50 C 10 20 30 40 50 µM MD
kDa
200
97
69
46
30
14
HO
b)

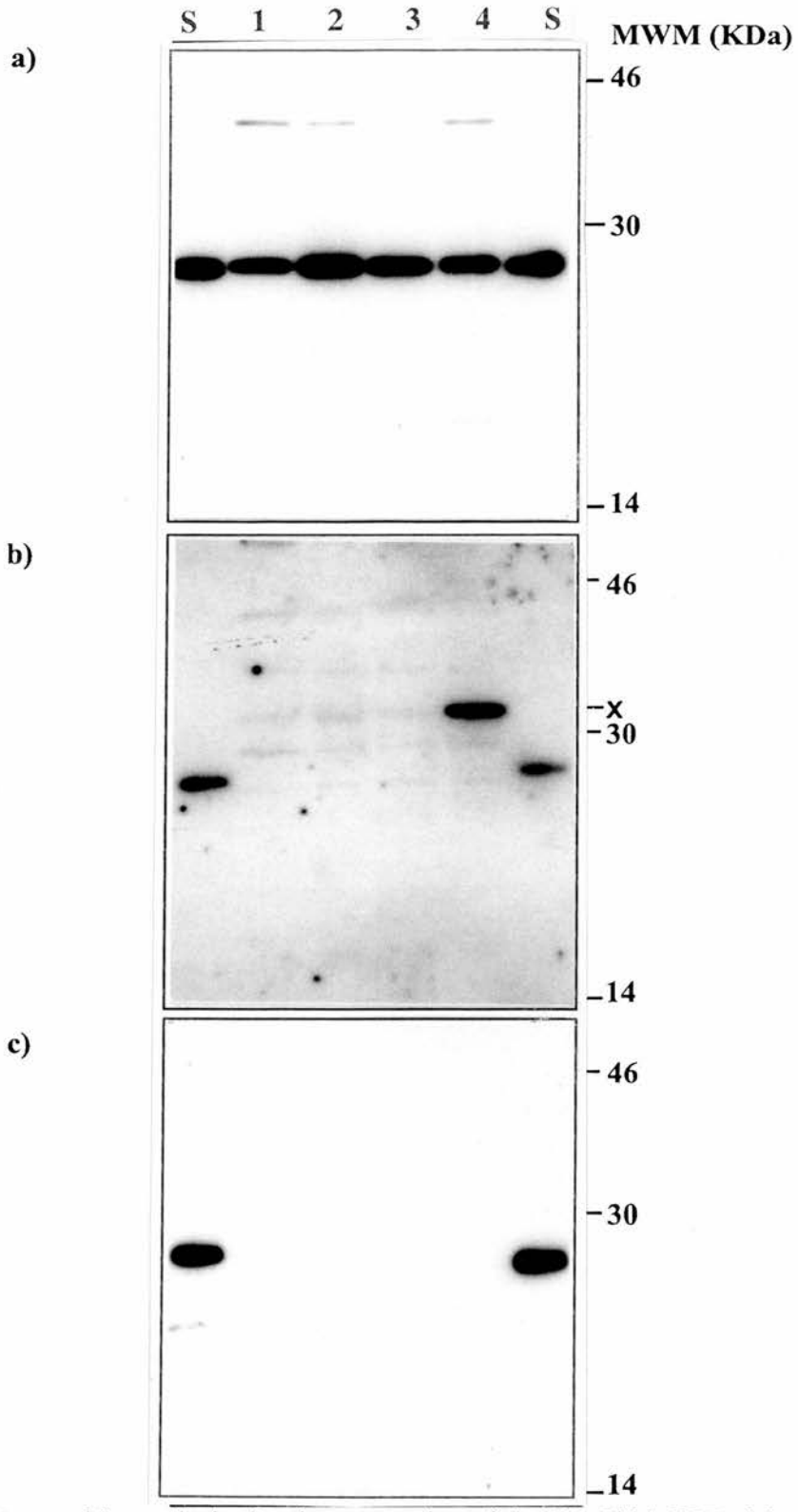
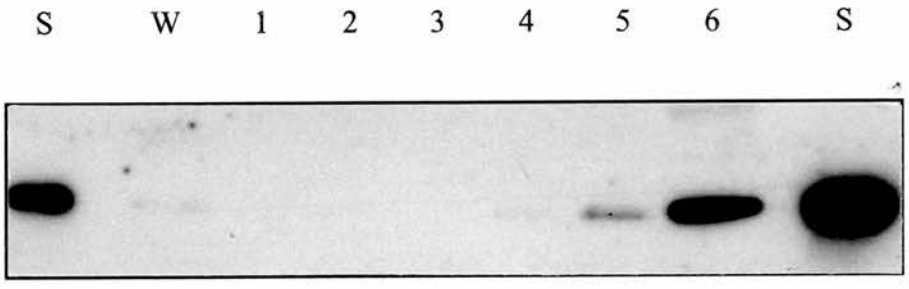


Figure 5.12 Western blot analysis showing expression of a) GST-Pi b) GST alpha and c) GST mu in 1, CHO-K1; 2, MRc20; 3, MRc30; 4, MRc40. 30µg of protein were loaded in tracks 1-4 and samples run on a 12% SDS-PAGE gel. S, purified protein standard; MWM, molecular weight markers. The band "x" in lane 4 of (b) is thought not to be GST mu but due to cross reaction of the antibody with another protein, possibly a different GST isozyme (J. Hayes, personal communication).

Figure 5.13 Western blot showing protein expression of GST-Pi in EJ cell lines.

S, Purified human GST-Pi; W, EJ-WT; 1, EJ-MRs40; 2, EJ-MRs60; 3, EJ-MRs80; 4, EJ-MRc20; 5, EJ-MRc30; 6, EJ-MRc40.
30μg of cytosolic protein were loaded in each lane. Samples were run on a 12% SDS-PAGE gel. Cells continuously exposed to menadione (EJ-MRc20, -30 and -40) but not those selected by pulse treatments with drug ((EJ-MRs40 and -60) showed induction of GST-Pi.



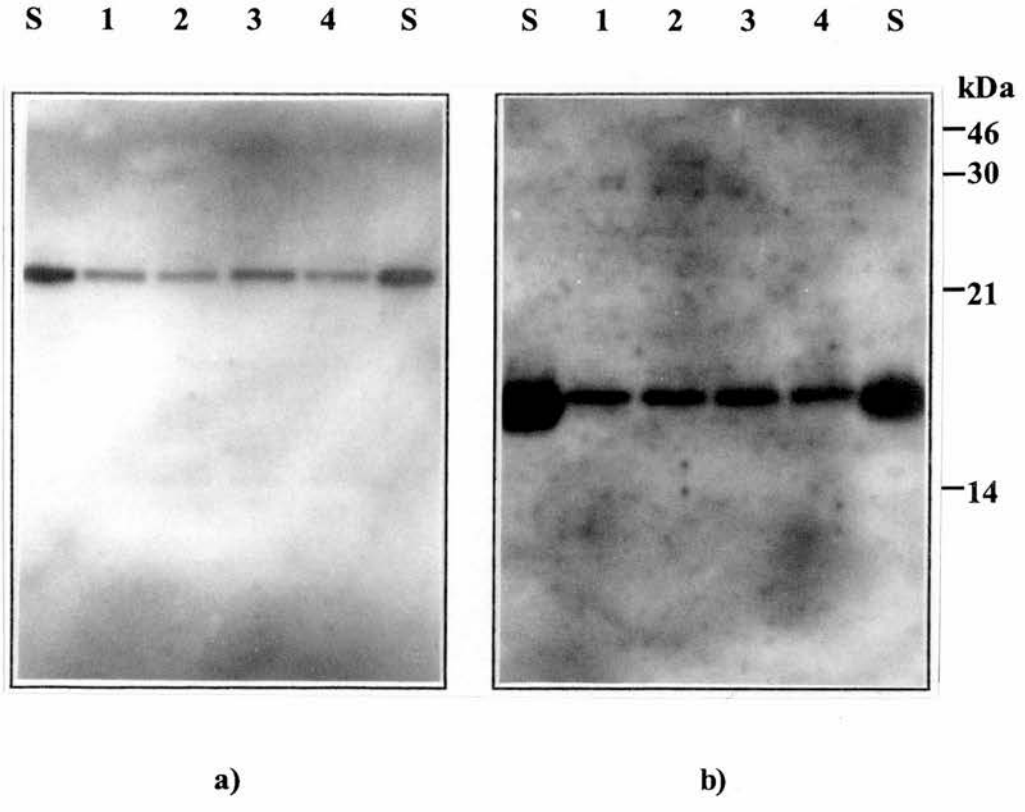


Figure 5.14 Western blot showing expression of **(a)** catalase and **(b)** Cu/Zn superoxide dismutase in CHO cell lines.

MWM, Molecular weight markers,
S, Standard purified protein,
1, CHO WT; 2, CHO-MRc20; 3, CHO-MRc30; 4, CHO-MRc40
30μg protein were loaded per track and samples were run on 15% SDS-PAGE gels.

5.3.5 Enzyme activities

Enzyme assays were performed on CHO-K1 and CHO-MRc40 cell lines according to the methods described in section 2.11. The results are summarised in Table 5.1.

Table 5.1. *Low molecular weight thiol content and glutathione-dependent enzyme expression in CHO-K1 and CHO-MRc40 cell lines.*

	CHO-K1	CHO-MRc40	Fold difference in activity [c]
Total GSH [a]	6.0 ± 0.2 (4)	12.1 (0.6) ***	2.0
Total cysteine [a]	2.5 ± 0.2 (4)	7.9 (0.5) ***	3.2
GST (CDNB) [b]	483.4 ± 155.0 (6)	726.8 (13.6) *	1.5
tGSHPx (CuOOH) [b]	53.1 ± 3.2 (6)	79.5 (4.9) ***	1.5
sGSHPx (H_2O_2) [b]	26.1 ± 8.5 (6)	41.5 (3.7) **	1.6
γ-GT [b]	2.6 ± 0.2 (4)	3.9 (0.2) *	1.5
GS [b]	4.5 ± 0.07 (4)	4.3 (0.04)	1.0
γ-GCS [b]	71.6 ± 4.1 (4)	53.6 (12.2)	0.8
GR [b]	210 ± 3.1 (4)	237 (10.8)	1.1

CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; tGSHPx, total glutathione peroxidase; sGSHPx, selenium-dependent glutathione peroxidase; γ-GT, γ–glutamyltranspeptidase; γ-GCS, γ-glutamylcysteine synthetase; GR, glutathione reductase; GS, glutathione synthetase.

[a] Mean values ± SD are expressed as nmol per mg protein.

[b] Mean values ± SD are expressed as nmol/min per mg protein

The numbers in parentheses represent the number of replicate samples

[c] $\frac{\text{Mean value for CHO-MRc40 activity}}{\text{Mean value for CHO-K1 activity}}$

Significant differences between CHO-MRc40 and CHO-K1 (unpaired t-test) are shown as * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

QAO was also measured and was found to be elevated 1.5-fold in CHO-MRc40 compared with CHO-K1 (153.9 and 105.8 nmol/min/mg protein, respectively; n=2).

5.4 DISCUSSION

There is now an enormous body of published work on the role of the glutathione-dependent enzymes in drug resistance. Overall GST activity is frequently increased in cell lines selected for resistance to different antineoplastic agents (Batist *et al*, 1986; Buller *et al*, 1987; Teicher *et al*, 1987; Lewis *et al*, 1988b). The increased expression of GST-Pi seen so frequently in models of drug resistance has aroused most interest (Black and Wolf, 1991). Induction of the pi isoenzyme of GST due to transcriptional activation of the gene is seen in all three of the CHO menadione-resistant sublines that were isolated. There was also a 2-fold increase in the amount of GST-Pi protein in the menadione-resistant cells and a 1.8-fold increase in GST activity. Although it has not been shown to be a GST-Pi substrate, elevated levels of this protein could increase the detoxification of menadione which is known to form a glutathione-conjugate (Nickerson *et al*, 1963). Although the product of conjugation of menadione with glutathione, thiodione, is thought to participate in redox-cycling and so is potentially toxic in its own right, it is more easily transported from the cell than menadione itself (Wefers and Sies, 1983). Perhaps a more important function of GST in protection from menadione cytotoxicity is to catalyse the formation of mixed disulphides between glutathione and protein thiol groups. Bellomo *et al* (1987) have reported a linear relationship between cellular glutathione level and the formation of mixed disulphides catalysed by thiol transferases. The protection of essential proteins from oxidative attack by the action of GST may explain why cells with constitutively elevated GST levels have a survival advantage over those that do not. The increased level of GST-Pi expression did not closely parallel the fold resistance in the cell lines indicating that other factors are involved in the resistance phenotype. Indeed, it is not expected that a single protein can protect cells from the diverse group of compounds to which the CHO-MRc40 cell line shows resistance.

It is not clear whether the expression of the GST-Pi isozyme directly contributes to drug resistance. In order to define the functional role of the GSTs in drug-resistance two groups have employed the strategy of transfection of the GST-Pi gene into mammalian cells. Nakagawa *et al* (1990) constructed an expression vector containing a cDNA for GST-Pi together with the β actin gene promoter and transfected this into NIH-3T3 cells. Overexpression of the transcribed and translated gene product was demonstrated. It provided a degree of protection against ethacrynic

acid and adriamycin in the transfected cells. Since the intracellular concentration of glutathione was unaffected by transfection the observed increase in resistance was considered to be a result of GST activity alone. However, the cells were still sensitive to the alkylating agents cisplatin, and chlorambucil and to ionising radiation. Moscow *et al* (1989) have carried out similar work. Two eukaryotic expression vectors containing the human GST-Pi cDNA together with either the CMV immediate early promoter or MTIIa were constructed. This vector was cotransfected with psv2neo into drug sensitive MCF-7 human breast cancer cells. Transfected cells were selected for G418 resistance and clones screened for GST activity. GST activity was elevated 15-fold. The selected clones showed 1.3 to 4.1 fold resistance to benzo(a)pyrene and 3.1 to 4.4 fold resistance to ethacrynic acid. There was no cross-resistance to adriamycin, melphalan or cisplatin.

More evidence that the GSTs play a role in drug-resistance is provided by a model of cross-resistance to multiple toxic agents in which GST-Pi is strikingly elevated. In the Solt-Farber model of hepatocellular carcinogenesis (preneoplastic liver nodules) cells develop resistance to the toxic effects of the carcinogen that induced malignant transformation and also cross-resistance to the cytotoxic effects of other structurally unrelated compounds (Farber, 1976). These nodules have markedly raised GST-Pi compared with normal liver (Kitahara *et al*, 1984). The observed overexpression of GST isoenzymes may give the preneoplastic foci a growth and hence selective advantage over surrounding tissue.

GSTs have also been implicated in the cellular response to ionising radiation and this raises the question as to whether the modest degree of radioresistance of menadione-resistant cells in anaerobic conditions might in part be due to alteration in GST expression. Whelan *et al* (1989) found an increase in GST-Pi after fractionated irradiation of MCF-7 cells. This cell line also showed 5-fold resistance to vincristine and 3-fold resistance to VP-16. This is not a universal phenomenon, as a SuSa cell line (a human testicular teratoma) treated in exactly the same manner with fractionated radiotherapy showed no significant alterations in GST isozyme profiles. In neither case was there any change in glutathione or glutathione peroxidase activity. Cholon *et al* (1992) examined the radiation response of several Chinese hamster ovary cell lines that had differing expression of the alpha and pi classes of GST. All of the cell lines had been isolated from the parental cell line, CHO-K1. There was no difference in survival after irradiation compared with the parental line. One of the CHO cell lines, RI, was obtained after treatment with the mutagen, ethyl methane sulphonate. This line possessed one-third normal alpha GST activity but a 10-fold increase in GST-Pi activity. It was found that GST-Pi expression diminished by 80%

in RI compared with control cells one hour after a dose of radiation sufficient to reduce the surviving fraction by 37%. Similarly Adams *et al* (1985) found that GST activity fell *in vivo* in mouse bone marrow following irradiation (2Gy). These studies suggest that GST activity is affected by ionising radiation, but whether it serves a protective function is as yet unknown.

Glutathione peroxidase is a seleno-enzyme present mainly in the cytosol and also, to a lesser extent, in the mitochondria. It metabolises hydrogen peroxide as well as organic hydroperoxides (Flohe, 1982). It is thought to be particularly important in the protection of lipid membranes from oxidative damage. Since it protects cells from hydrogen peroxide, one of the products of menadione-metabolism, it is not surprising that a modest increase in expression of GSHPx is seen in the more resistant of the menadione-resistant cell lines. The fold increase in GSHPx activity in the CHO-MRc40 cell line compared with CHO-K1 is 1.8 fold. The ratio of the activity of total GSHPx to selenium-dependent GSHPx activity is 2.0 and 1.9 for the CHO-K1 and CHO-MRc40 cell line respectively. These data indicate that both selenium-dependent and -independent GSHPx activities are increased in the menadione-resistant cell lines and the percentage change in the two types of activity is similar. Increased selenium-independent GSHPx activity is consistent with elevation of GST in these cells because GSHPx activity is expressed by the alpha and to a lesser extent by the Pi class of GST (Singh *et al*, 1985; Stockman *et al*, 1984). Several studies have examined the expression of selenium-independent GSHPx and selenium-dependent GSHPx in matched normal and tumour tissue (Di Ilio, *et al*, 1987; Carmichael, *et al* 1988b). In all cases, significant elevation (of about 2 fold) in selenium-independent GSHPx and selenium-dependent GSHPx were observed in tumour tissue as compared with the normal tissue. This suggested that GSHPx may contribute to intrinsic drug-resistance in certain types of tumour.

Others have reported elevation of GSHPx in drug-resistant cell lines. For example, this has been noted in adriamycin-resistant cell lines described by Singh *et al* (1989). The adriamycin-resistant cell line contained 12 to 14 fold more GSHPx than adriamycin-sensitive cells. Subcellular fractions were obtained from both cell lines and free radical formation in the presence of adriamycin was measured by using electron spin resonance spectroscopy. In the presence of adriamycin, adriamycin-semiquinone free radical, superoxide anion and ·OH were detected in mitochondria, microsomes and nuclei. The mitochondria and nuclei of adriamycin-resistant cells showed an overall two fold decrease in formation of oxyradicals. Formation of free radicals was decreased by the addition of purified GSHPx in a dose-dependent manner. The decreased free radical formation in adriamycin-resistant cells was

therefore a result of detoxification of hydrogen peroxide by GSHPx.

Glutathione reductase catalyses the NADPH-dependent regeneration of reduced glutathione. Transcriptional activation of the glutathione reductase gene was not seen in any of the three CHO menadione-resistant sublines. Neither was elevation of its mRNA seen in response to transient oxidative stress in either cell line. This lack of inducibility of glutathione reductase was also reflected in the enzyme activity which was unchanged in the drug-resistant cell line compared with CHO-K1. Although elevated levels of glutathione are a common feature of drug-resistance, elevation of glutathione reductase activity is rare (Mansouri *et al*, 1989). It seems that in most cases where glutathione concentration is elevated, the increase is due to enhanced synthesis or transport of the components of glutathione into the cell rather than to the restoration of GSH from GSSG by glutathione reductase activity (Anderson and Meister, 1977). There is evidence that the activity of glutathione reductase is limited in the presence of menadione because of depletion of reduced pyridine nucleotides. Also the GSSG formed as a result of menadione metabolism and which is the substrate for glutathione reductase is rapidly excreted from the cell (Bellomo *et al*, 1987).

There was no evidence for transcriptional activation of the gene encoding γ-GCS either in response to transient stress or as a feature of the menadione-resistant cells. One group has reported enhancement of γ-GCS mRNA in response to oxidative stress (Woods *et al*, 1992) and γ-GCS activity is sometimes elevated in models of resistance to alkylating agents (Black and Wolf, 1991). In the former study, the kidneys of rats given prolonged treatment with mercury hydroxide showed both elevation of glutathione and γ-GCS mRNA. Another group has reported increased activity of γ-GCS in a L-PAM-resistant murine leukaemia cell line in which glutathione was elevated (Ahmad *et al*, 1987). However, the mechanism of γ-GCS regulation was not investigated. Although increased expression of γ-GCS may occur as a result of oxidative stress, this does not appear to be a constant feature and was not observed in our model.

The physiological function of heme oxygenase is to catabolise heme, a reaction that is accompanied by the release of iron. During oxidative stress, HO is induced and so the amount of free intracellular iron increases. Vile *et al* (1993) have recently demonstrated that ferritin, the main storage protein for iron, is induced by oxidative stress and that this induction is dependent on HO. The enhancement of ferritin activity results in increased sequestration of iron and so prevention of its use in iron-catalysed free radical reactions such as the Fenton reaction in which iron catalyses the decomposition of hydrogen peroxide with the formation of the hydroxyl anion

(Halliwell and Gutteridge, 1984). It has also been shown that preirradiation of human skin fibroblasts with UVA protects them from the effects of a subsequent treatment. Evidence was presented that this adaptive response was mediated by HO and ferritin (Vile *et al*, 1994).

The basal level of HO mRNA is higher in menadione-resistant than sensitive cells. It is possible to speculate that, compared with parental cells, there would be a relative paucity of free iron in menadione-resistant cells in the unstressed state. This would result in fewer free radicals being formed immediately after exposure to oxidative stress. Furthermore, the greater availability of glutathione in these cells would result in more efficient scavenging of the radicals that were formed. Given that the MRc40 cells are able to grow in 40μM menadione and that the concentration of menadione used in the transient stress experiment shown in Figures 5.9 was only 20μM, it is likely that the redox state of the menadione-resistant cells was never significantly disturbed. This may explain the relative lack of accumulation of HO in these cells. In support of this possibility Applegate *et al* (1991) observed that lowering glutathione levels substantially enhances the maximum induction of HO mRNA. They noticed that the fold increase in HO mRNA was lower in primary epidermal keratinocytes than in fibroblasts cultured from the same foreskin biopsy. This was attributed to a 2 to 3 fold higher glutathione level in keratinocytes. Keyse and Tyrrell (1987) found that cells that were first depleted of glutathione required significantly less UV light exposure for HO induction.

Western blot analysis carried out to examine the expression of Cu/Zn superoxide dismutase and catalase in the CHO cells lines showed there was no difference between the menadione-resistant compared with the menadione-sensitive cell lines. Much work has been performed to elucidate the role of these two enzymes in defence against oxidative stress with some contradictory results. The complete absence of SOD activity sensitises *E. coli* (Natvig *et al*, 1987) and drosophila (Phillips *et al*, 1989) to oxidative stress. In addition, there are several examples in which an increase in SOD has been shown to provide protection against oxidative stress (Elroy-Stein *et al*, 1986; Kyle *et al*, 1988). However there are exceptions. For example, a large increase in SOD sensitised *E. coli* to paraquat (Bloch and Ausubel, 1986) and inactivation of Cu/Zn-SOD in human fibroblasts increased their growth rate (Michiels *et al*, 1988).

A lack of responsiveness of SOD and catalase to oxidative stress has been reported by others. In order to examine the effect of oxidative stress on the expression of superoxide dismutase Strålin and Marklund (1994) treated human dermal fibroblasts with a variety of oxidising agents. Paraquat, α-naphthoflavone,

hydroquinone, buthionine sulphoximine, cumene hydroperoxide and high partial pressure of oxygen were among the agents tested. There was no induction of Cu/Zn-SOD activity by any of the oxidative treatments. Indeed, there was dose-dependent reduction of Cu/Zn-SOD activity by many of them. Similarly, there were absent or only modest inductions of Mn-SOD activity by the oxidants. This is in marked contrast to the major induction of Mn-SOD produced by cytokines (Wong and Goeddel, 1988). Shull *et al* (1991) demonstrated a differential response of SOD and catalase according to the inducing agent. They examined the *in vitro* effect of two oxidant stresses, H_2O_2 and a xanthine/xanthine oxidase generating system, on the levels of steady state mRNA of Mn-SOD, Cu/Zn-SOD, GSHPx and catalase in hamster tracheal epithelial cells. After exposure to xanthine/xanthine oxidase, Mn-SOD mRNA was selectively increased in a dose- and time-dependent manner. Catalase, GSHPx and Cu/Zn-SOD were all unaffected. The addition of H_2O_2 caused a dose-dependent increase in catalase mRNA while having only a minor effect on transcription of the SOD and GSHPx genes. The ability of H_2O_2 to induce catalase was also demonstrated by Spitz *et al* (1988) who isolated stable, H_2O_2-resistant variants from a Chinese hamster fibroblast cell line. These isolates showed increased catalase activity (up to 50-fold). There was strong correlation between the fold increase in catalase and the degree of resistance to H_2O_2 suggesting a phenomenological link between the two. It is clear, however, from the present work that increased catalase activity is not essential for H_2O_2 resistance. The menadione-resistant cell line, CHO-MRc40, shows cross-resistance to hydrogen peroxide but no alteration in catalase expression. Interestingly, catalase (but not SOD) activity was elevated in the menadione resistant Chinese hamster fibroblasts cell-lines selected for menadione-resistance by Martins *et al* (1991). Catalase activity was elevated 2.8-fold in these cells.

Amstad *et al* (1991) studied the individual roles of Cu/Zn-SOD and catalase by transfecting human cDNAs encoding these proteins into mouse epidermal cells. Interestingly, two transfectant clones that overproduced Cu/Zn-SOD by 2.3 and 3.6 fold were hypersensitive to the formation of DNA single-strand breaks, growth retardation and killing by an extracellular burst of superoxide plus H_2O_2. On the other hand, catalase overproducing transfectants were protected compared to the parent clone. Interestingly, an increase in Cu/Zn-SOD was tolerated without an increase in oxidant sensitivity when it was counterbalanced by a corresponding increase in catalase in a double transfectant. It was concluded that the balance of Cu/Zn-SOD and catalase is more important for the overall sensitivity to oxidative stress than the absolute levels of the two. The imbalanced expression of SOD and

catalase activities within cells may be deleterious perhaps through the overproduction of peroxides. That overexpression of SOD can actually be detrimental was illustrated by the work of Bloch and Ausubel (1986). *E. coli* isolates carrying a multicopy plasmid containing the Mn-SOD gene, *sodA*, were more sensitive than the wild type to paraquat-mediated growth inhibition. The transfectants were twice as sensitive to paraquat as the wild type cells and this was despite a five-fold increase in SOD activity in the former. Eukaryotic cells transfected with a recombinant plasmid bearing the human Cu/Zn-SOD gene have also been studied (Elroy-Stein *et al*, 1986). They showed resistance to paraquat but interestingly those transfectants that moderately overproduced the enzyme activity (3.6-fold over the wild-type) were more resistant than controls, whereas those that maximally overproduced the activity (5.8-fold over the wild-type) demonstrated only baseline resistance to paraquat. No Cu/Zn-SOD overproducing isolates demonstrated decreased resistance.

The contribution of QAO to the protection against redox-active compounds has often been assessed in experimental models by inhibiting QAO with dicoumarol to potentiate cytotoxicity (Thor *et al*, 1982; Wefers and Sies, 1983) or by correlating 3-methylcholanthrene-mediated induction of QAO with protection from quinone toxicity (Talalay *et al*, 1988). A purely protective role for QAO has been questioned (Talcot *et al*, 1983). The use of dicoumarol has been reported to increase the cytotoxicity of menadione in phenobarbital-pretreated hepatocytes - suggesting a shift toward the one electron reductive pathway takes place (Powis *et al*, 1987). QAO has a 10-fold greater Km than NADPH cytochrome P450 reductase for menadione (Thor *et al*, 1982). Therefore, only a slight increase in QAO activity may be physiologically significant (Powis, 1981).

Liu *et al* (1993) used cell lines derived from the livers of newborn mice and these were examined for differences in menadione toxicity. The c^{14Cos}/c^{14Cos} mouse (unlike the c^{ch}/c^{ch} mouse) has a homozygous deletion of about 1.2cm of chromosome 7. The untreated c^{14Cos}/c^{14Cos} newborn exhibits a marked transcriptional activation of the hepatic QAO gene (*nmo*-1) when compared with c^{ch}/c^{ch} (wild-type) and c^{ch}/c^{14Cos} (heterozygotes). This gene was also activated in SV40-transformed cell lines that were established from the c^{14Cos}/c^{14Cos} mice (14cos/14cos). 14cos/14cos cells also have a 3-fold elevation of GSH and 50% increase in GSTA1, UDP-glucuronyl transferase and Cu/Zn-SOD activities and a decrease in catalase of 6-fold. 14cos/14cos cells, therefore, permanently exhibit an oxidative stress response. 14cos/14cos cells were 2 to 4 times more resistant to menadione than ch/ch cells (derived from wild-type newborn liver). Even when they were treated with dicoumarol (to block QAO) 14cos/14cos cells show a 2-fold resistance to menadione.

Cellular resistance to menadione correlated well with total GSH concentration. In fact no cytotoxicity was detectable as long as GSH concentration was maintained at 5-8nmol/mg protein. These results are consistent with the dominant role of GSH-depletion in mediating menadione toxicity and QAO appears to play a relatively minor role. In the menadione-resistant cells described in this thesis there was only minor alteration in QAO activity.

5.5 SUMMARY

In this chapter the further characterisation of menadione-resistant variants of the parental cell lines, CHO-K1 and EJ-WT, has been recorded. Intriguingly, many of the changes observed in the drug-resistant cell lines appear to be the same as those observed after transient oxidative stress in wild-type cells. Induction of GST-Pi, GSHPx and heme oxygenase were all seen after transient oxidative stress in CHO-K1 and these same genes appear to be constitutively overexpressed in menadione-resistant cells. There is, therefore, an apparent immortalisation of an adaptive response to oxidants in menadione-resistant CHO cell lines. This could be explained by mutation of a gene that regulates the adaptive response to oxidative stress in these cells.

CHAPTER SIX

THE ROLE OF NF-κB IN MENADIONE RESISTANCE

6.1 INTRODUCTION

NF-κB is a eukaryotic inducible transcription factor which up-regulates the expression of several inflammatory and immune response genes including interleukins-1, -6 and -8, interferon-β and tumour necrosis factor-α (TNF-α) as well as a variety of cell adhesion molecules. Sen and Baltimore (1986) showed that NF-κB is inducible in a variety of different cell lines, including HeLa and Jurkat lines, and not just in pre-B lymphocytes as was originally thought. NF-κB is required for the replication of several viruses, including HIV-1, and stimulates expression of HIV-1 genes through binding to two inducible sites in the viral long terminal repeat (LTR) promoter. NF-κB DNA-binding is induced by a wide variety of stimuli including phorbol esters, inflammatory cytokines, UV radiation, ionising radiation, viral and bacterial proteins, lipopolysaccharides, double-stranded RNA and reduced protein synthesis. It exists as various multiprotein complexes. The simplest DNA-binding form of NF-κB is composed of dimerised 50kDa protein (p50) which itself is derived from a heterodimeric complex of NF-κB containing p50, two p65 non-binding subunits and the inhibitory polypeptide, IκB. NF-κB exists in the cytoplasm of unstimulated cells. When cells are stimulated with phorbol esters or cytokines the NF-κB complex undergoes post-translational modification. IκB is phosphorylated through the action of protein kinase C (PKC) or other kinases (Ghosh and Baltimore 1990). This phosphorylation dissociates NF-κB from IκB and allows NF-κB to translocate to the nucleus where it activates its target genes. NF-κB can very rapidly activate genes because the NF-κB protein is already present in unstimulated cells and only requires the release of IκB for its activation. Not all activation of NF-κB is mediated by PKC since depletion of PKC by chronic PMA treatment does not affect NF-κB activation by TNF-α. NF-κB binds to a decameric sequence motif in promoter or enhancer elements.

It has been proposed that the formation of reactive oxygen species may be a common feature of the diverse NF-κB-inducing signals. The redox regulation of NF-κB has received much attention because it is an essential activator of human

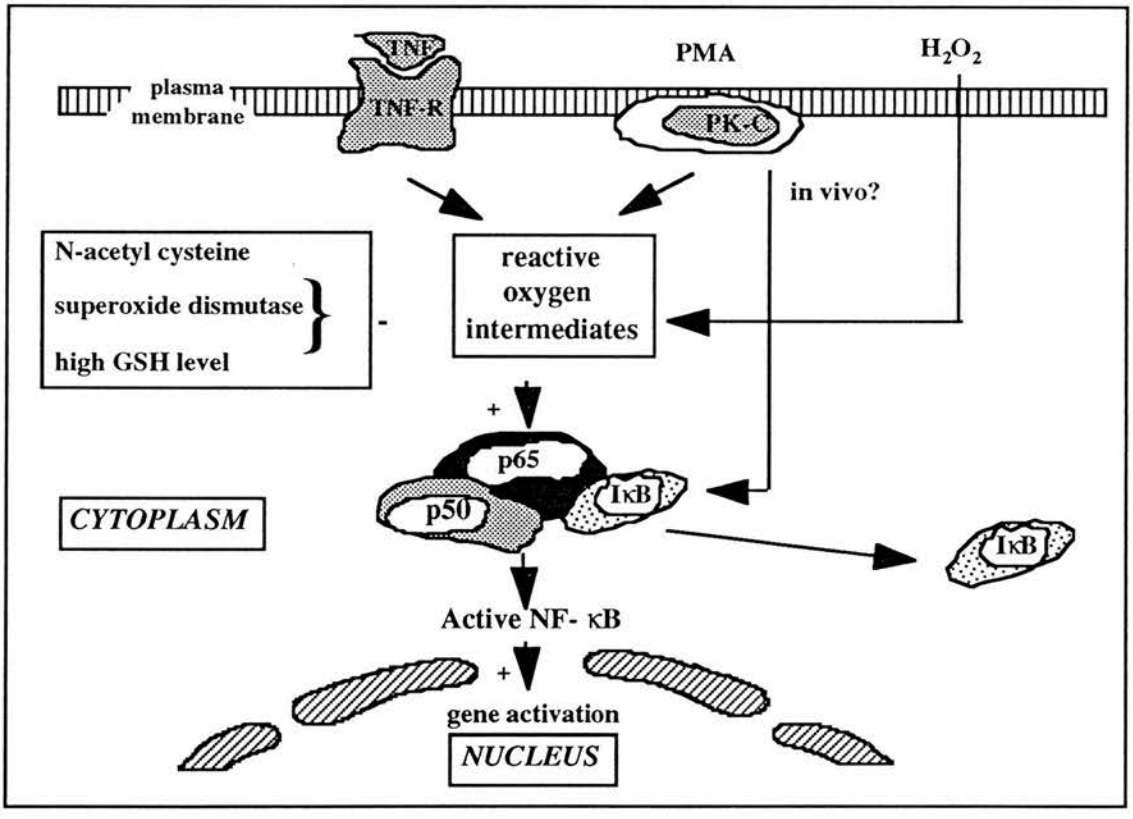
immunodeficiency virus replication and therefore redox status may play a crucial role in the pathogenesis of the Acquired Immune Deficiency syndrome. The proposed scheme by which NF-κB is activated by reactive oxygen species is shown in Figure 6.1. Meyer *et al* (1993) studied the effect of cellular redox status on NF-κB activation in HeLa cells that had been transiently transfected with CAT reporter constructs driven by a thymidine kinase (TK) promoter under the control of two NF-κB binding sites. The addition of 200μM H_2O_2 led to an increase by 30-fold of the CAT protein. The same experiment in Jurkat cells gave similar results. In the case of Jurkat cells 30μM H_2O_2 was sufficient to cause strong transactivation of an HIV-1 LTR driven CAT reporter construct. The results in HeLa cells were confirmed using electrophoretic mobility shift assays in which κB-binding activity showed a dramatic increase upon treatment of cells with 100 to 250μM H_2O_2. Hydrogen peroxide also potentiated the activation of NF-κB in response to PMA. Treatment with 120nM PMA caused a 60-fold increase in NF-κB activation whereas the combination of H_2O_2 and PMA caused a 120-fold induction of CAT protein. Similar results were obtained with Jurkat cells.

Meyer *et al* also studied the effect of antioxidants on the activation of NF-κB. The metal chelator and radical scavenger, pyrrolidine dithiocarbamate (PDTC), at a concentration of 60mM interfered with activation of NF-κB by PMA. Staal *et al* (1990) showed that intracellular thiol levels play a key role in regulating DNA-binding by NF-κB. Stimulation with TNF-α and/or PMA activated NF-κB but depleted intracellular glutathione. *N*-acetyl-L-cysteine prevented this depletion of thiol and at the same time blocked activation of HIV LTR by NF-κB.

NF-κB appears to act primarily as an oxidative stress responsive transcription factor. Since one of the major phenotypic changes associated with acquired menadione resistance is an alteration in glutathione metabolism, it was decided to study the activation of NF-κB in some of the cell lines described in this thesis.

Figure 6.1 *Activation of NF-κB by reactive oxygen species*

Proposed model showing the presumed involvement of oxyradicals in activation of NF-κB. Cytokines (represented here by TNF), PMA and H_2O_2 all generate reactive oxygen species which stimulate release of IκB from the NF-κB multiprotein complex. This allows translocation to the nucleus and gene activation. Various antioxidants have been shown to inhibit NF-κB activation. It is possible that PKC is able to activate NF-κB directly.



Based on Schreck (1991) EMBO J. **10**, 2247-2258.

6.2 STRATEGIES

To study the effect of oxidative stress on NF-kB activation, menadione-sensitive and -resistant cells were transiently transfected with chloramphenicol acetyl transferase (CAT) reporter constructs.

Oligonucleotides and plasmid constructs. pCAT-Promoter vector (Promega, Madison, USA) was cleaved with *Bgl*II. The pCAT-Promoter vector contains an SV40 promoter upstream from the CAT gene. The vector also contains the gene for ampicillin resistance. The cleaved vector was ligated with synthetic oligonucleotides (sequences shown below) encompassing three NF-κB binding sites (which are underlined) in the correct (1) or reverse orientation (2):

5' - GATCTGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCA - 3' (1)

5' - GATCTGGAAAGTCCCCAGCGGAAAGTCCCCAGCGGAAAGTCCCA - 3' (2)

Oligonucleotides were synthesised at the Imperial Cancer Research Fund laboratories, Clare Hall, Herts. Plasmids containing the above oligonucleotide (correct and reverse orientation) upstream of a CAT reporter gene were generated and designated pCATNF-κB$^+$ and pCATNF-κB$^-$ respectively. The orientation of the inserted fragments was confirmed by DNA sequencing. Menadione-sensitive (CHO-K1) and menadione-resistant (CHO-MRc40) cells were then transfected with either pCATNF-κB$^+$ or pCATNF-κB$^-$ according to the protocol outlined in section 2.8.1. Mock transfection and transfection with pCAT-Control and pCAT-Promoter vectors was carried out to provide control samples. pCAT-Control vector contains SV40 promoter and enhancer sequences upstream from the CAT gene.

Oxidative stress induction of NF-κB activity. Transiently transfected cells were allowed to recover for an overnight incubation at 37°C. They were then treated with oxidants as follows:
Experiment 1: 15μM menadione.
Experiment 2: 15, 25 or 35μM menadione or 50, 100 or 150μM H_2O_2.
Experiment 3: 100μM H_2O_2 or 100μM H_2O_2 plus 120nM PMA.

CAT assay. Cell extracts were prepared by three freeze-thaw cycles and the protein content measured by the method of Lowry. CAT assays were carried out according to the method described in section 2.8.2.

6.3 RESULTS

The results of experiments 1, 2 and 3 are shown in figures 6.2, 6.3 and 6.4 respectively.

Figure 6.2 *Effect of menadione on induction of NF-κB* (see page 170)

The upper panel shows the result for CHO-K1 and the lower panel for the drug-resistant cell line, CHO-MRc40. The band (a) represents non-acetylated chloramphenicol. Bands (b) and (c) represent modified forms of chloramphenicol that have been acetylated at one or the other of two potential sites. As expected, the mock transfected cells show no CAT activity (CAT being a uniquely bacterial gene). The pCAT-Control vector resulted in moderate expression of CAT activity. In the case of CHO-K1, pCAT-Control activity appeared greater in menadione-treated than in untreated cells. This could be explained by a difference in transfection efficiency. The pCAT-Promoter lacks an enhancer sequence and does not give rise to detectable CAT activity. Both pCATNF-κB$^+$ and pCATNF-κB$^-$ resulted in strong CAT activity in both cell lines. That the CAT activity is stronger for pCATNF-κB$^+$ and pCATNF-κB$^-$ than for pCAT-Control indicates that NF-κB is an efficient enhancer of the CAT gene. The lack of effect of menadione on CAT activity suggests that in this model oxidative stress does not induce NF-κB DNA-binding.

Figure 6.3 (a) *Effect of different concentrations of menadione and H_2O_2 on induction of NF-κB in CHO-K1 (see page 171)*

As in Figure 6.2, there was no observed increase in CAT activity in CHO-K1 cells following treatment with menadione (panel b) even when high concentrations of the quinone are used. Similarly, there is no increase in CAT activity in CHO-K1 cells following treatment with H_2O_2 at various concentrations (panel c). Indeed, the highest concentration of menadione (35μM) and all three concentrations of H_2O_2 appear to decrease CAT activity compared with the controls (panel a). This may be because these concentrations of oxidant cause such severe toxicity that transcription is inhibited.

Figure 6.3 (b) *Effect of different concentrations of menadione and H_2O_2 on induction of NF-κB in CHO-MRc40 (see page 172)*

The result is essentially the same as for CHO-K1 except that in the drug-resistant cell line there is less inhibition of CAT activity at the highest menadione concentration and following treatment with H_2O_2.

Figure 6.2

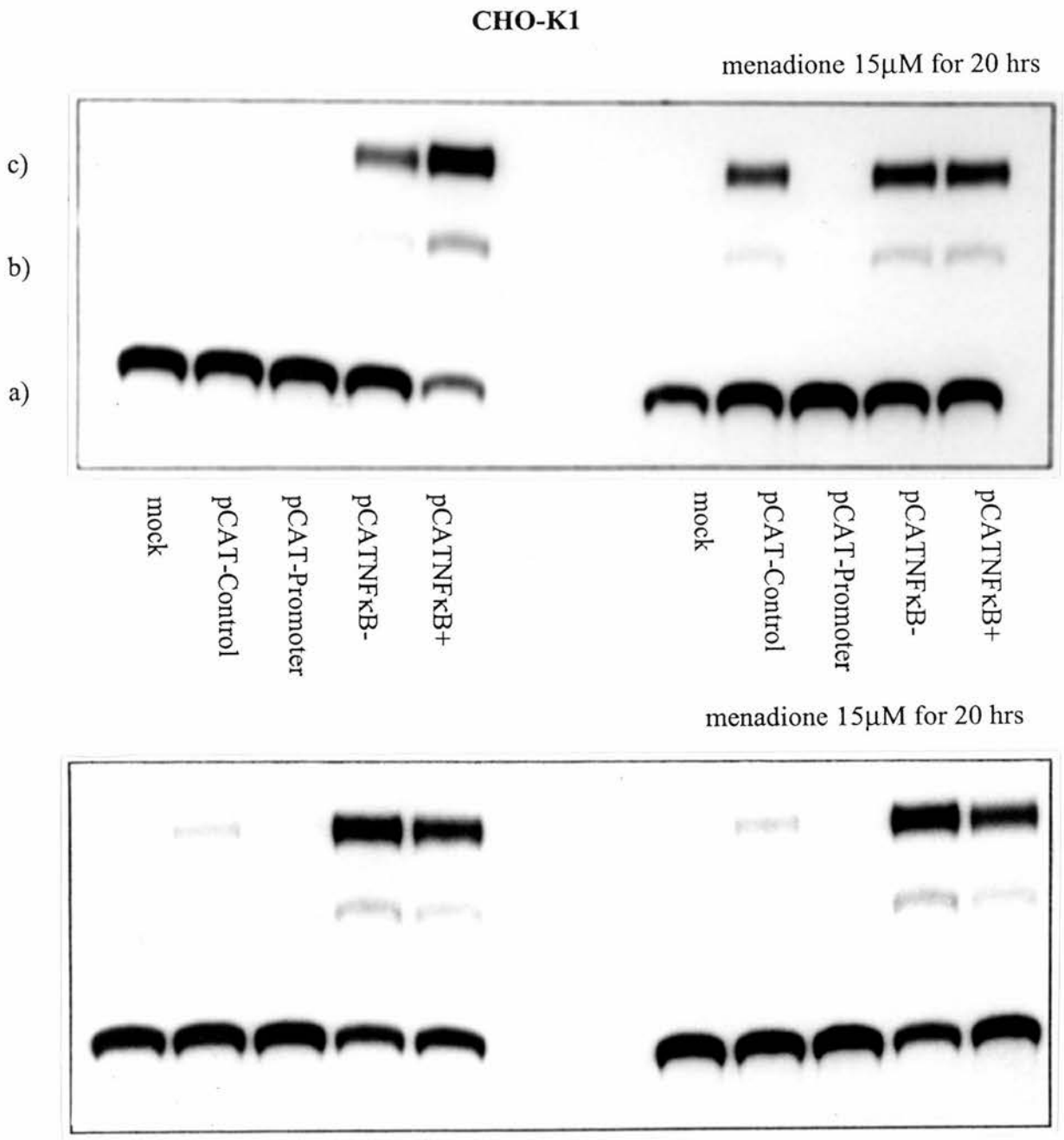

CHO-K1
menadione 15μM for 20 hrs
c)
b)
a)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB-
pCATNFκB+
mock
pCAT-Control
pCAT-Promoter
pCATNFκB-
pCATNFκB+
menadione 15μM for 20 hrs
CHO-MRc40

Figure 6.3 (a)

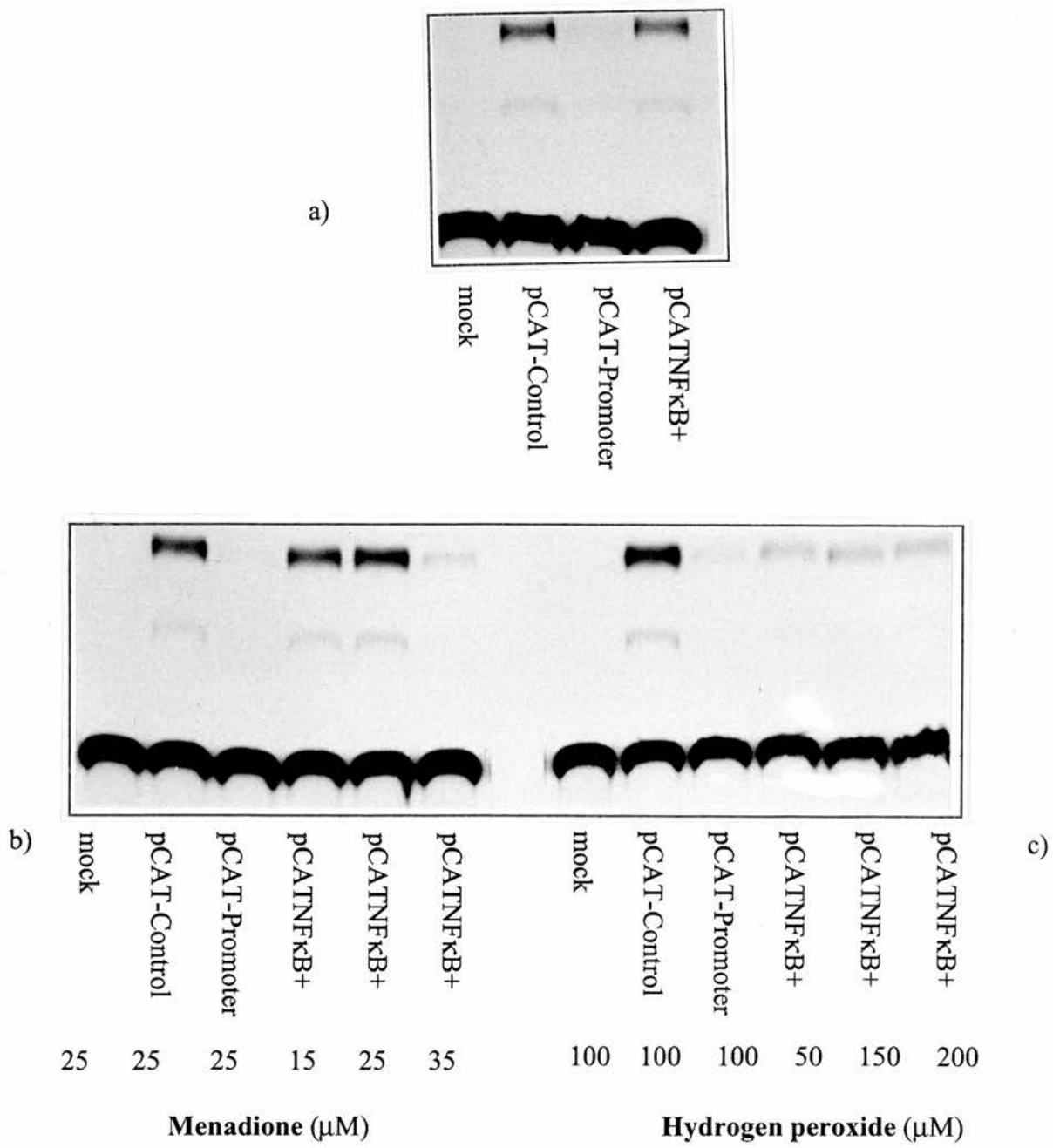

a)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
b)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
pCATNFκB+
pCATNFκB+
25
25
25
15
25
35
Menadione (μM)
c)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
pCATNFκB+
pCATNFκB+
100
100
100
50
150
200
Hydrogen peroxide (μM)

Figure 6.3 (b)

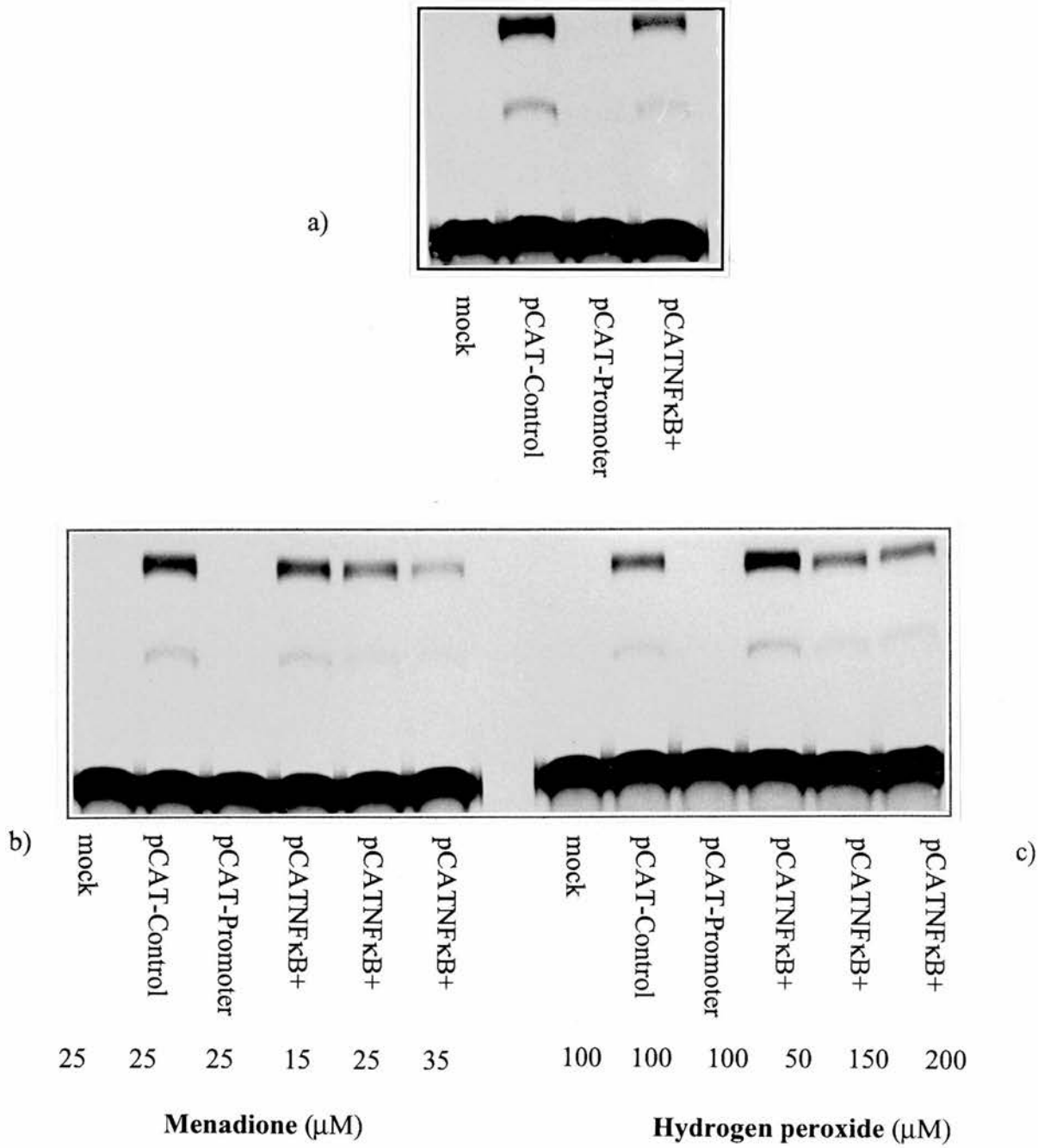
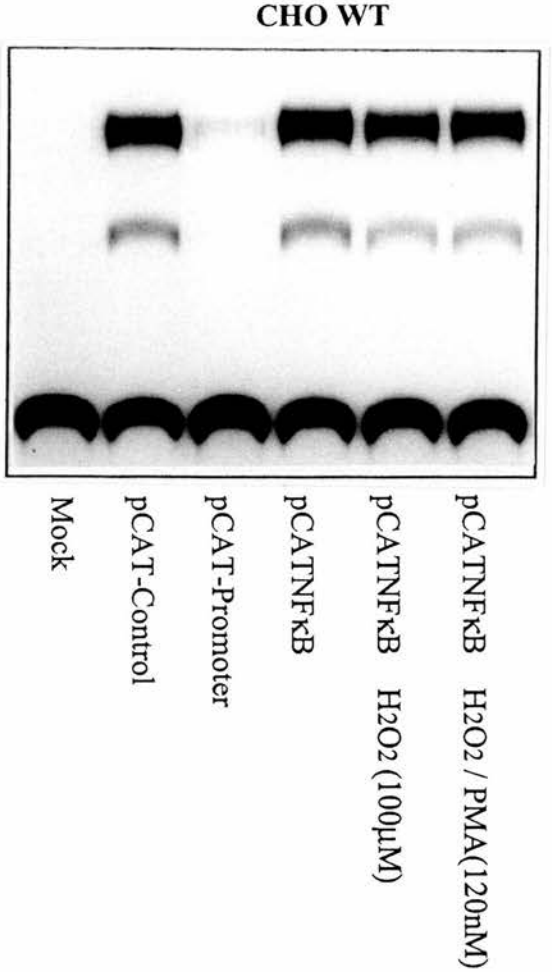

a)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
b)
c)
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
pCATNFκB+
pCATNFκB+
mock
pCAT-Control
pCAT-Promoter
pCATNFκB+
pCATNFκB+
pCATNFκB+
25 25 25 15 25 35
100 100 100 50 150 200
Menadione (μM)
Hydrogen peroxide (μM)

Figure 6.4 *Effect of H_2O_2 and the combination of H_2O_2 and PMA on induction of NF-κB* in CHO-K1.

CAT activity was not increased following treatment with H_2O_2 (100μM) or H_2O_2 (100μM) in combination with 120nM PMA compared with pCAT-Control transfectants.



6.4 DISCUSSION

Several studies published during the last few years indicate that oxyradicals play an important role in gene regulation. Different modes of redox regulation have been described for a variety of mammalian transcription factors. The NF-κB elements in regulatory domains of genes appear to serve specifically as response elements for oxidant stress. Cellular stress reactions such as heat shock and chemicals do not activate NF-κB.

Schreck *et al* (1991) have shown that the binding of NF-κB to DNA is profoundly influenced by the redox state of the cell. This has been shown for both B-cells and for an epithelial cell line (HeLa). Other workers including Brennan *et al* (1993) although able to repeat the same observations as Schreck in Jurkat T cells showed that H_2O_2 does not induce NF-κB in two other cell lines (EL4 murine T-cells and KB human epithelial cells). Furthermore, the prooxidant, diamide, instead of activating NF-κB, proved inhibitory. Brennan also showed that there was no inhibition of interleukin-1 induction of NF-κB by NAC in EL4 and KB cells. In other words the reactive oxidant model of NF-κB induction appears to be cell and possibly cytokine-specific.

The work described in this chapter suggests that NF-κB DNA-binding is not influenced by oxidative stress in CHO cells. This is true for both wild-type cells and for cells that have acquired resistance to menadione. Schreck *et al* (1992) showed that butyl peroxide, like H_2O_2, activates NF-κB but agents leading to the production of $O_2^{-\cdot}$, such as menadione, are not effective. Insofar as it is induced by peroxides but not by superoxide, the function of NF-κB is more analogous to bacterial OxyR than to SoxRS. It is perhaps not surprising that there is little difference in NF-κB activation in CHO-K1 and CHO-MRc40 in the resting state or following treatment with menadione. However, in contrast to the work of Schreck, there is no increase in activation of NF-κB in CHO cells stimulated by H_2O_2 or by the combination of H_2O_2 and PMA which was shown to cause "super-stimulation" in Jurkat cells.

6.5 SUMMARY

It has been proposed that NF-κB is primarily an oxidative-stress responsive transcription factor. Several researchers have shown that oxidants such as H_2O_2 and PMA are able to stimulate binding of NF-κB to DNA. This, however, has not been a universal finding and may be a cell and tissue-type specific phenomenon. There is no evidence to date that NF-κB activation is altered in menadione-resistant CHO cells.

This is despite an alteration in glutathione metabolism in the resistant compared to the sensitive cell lines.

CHAPTER SEVEN

CONCLUSION AND FUTURE WORK

Oxidative stress triggers cellular defence mechanisms that counteract reactive oxygen species and the damage they cause. They include the induction of enzymes with radical scavenging and repair activities. The oxidative stress response is well characterised in bacteria. Two oxyradical-responsive transcription factor systems (OxyR and SoxRS) have been investigated in detail. They control the expression of multiple antioxidant enzymes. The mechanisms and factors regulating oxidant and antioxidant responses in eukaryotic cells are poorly understood. In order to study the genetic changes that generate resistance to oxidants in eukaryotic systems, cell lines that are resistant to menadione have been isolated. Resistant cells were selected by continuous addition of menadione to the growth medium. Analysis of proteins by 2-dimensional gel electrophoresis revealed differences between menadione-resistant and -sensitive cell lines. Menadione-resistant cells exhibit cross-resistance to other oxidants and to heat and to ionising radiation in anaerobic conditions.

(a) Cystine transport

It became clear from HPLC and NMR studies that the metabolism of glutathione is altered in menadione-resistant cells compared with parental cells and that both GSH and cysteine are elevated in the former. Furthermore, resistant cells seem to have an enhanced capacity for resynthesis of GSH in response to oxidative stress. The mechanisms underlying this phenomenon have not been fully elucidated. The rate-limiting step in glutathione synthesis is catalysed by γ-GCS. However, the activity of γ-GCS is not altered in menadione-resistant cell lines nor is the level of γ-GCS mRNA increased. Increased activity of γ-GCS can not therefore explain the observed difference in thiol metabolism. Cysteine is known to regulate glutathione synthesis. The intracellular cysteine concentration is maintained by uptake of cystine, which is rapidly reduced to cysteine once it enters the cells. The intracellular glutathione concentration diminishes when cells are grown in cystine-free medium. What are the mechanisms by which menadione-resistant cells maintain a high level of cysteine? One possibility is the up-regulation of a specific cysteine transport system. An anionic amino acid transport system that is highly specific for cystine and glutamate has been described in various cells including cultured human

fibroblasts (Bannai and Kitamura, 1980) and has been designated " System x_c^-" (Bannai, 1986). Deneke (1992) studied bovine pulmonary artery endothelial cells and examined the mechanism of the induction of cysteine transport using sodium arsenite. Exposure of endothelial cells to subtoxic concentrations of arsenite resulted in a concentration-dependent increase in cystine uptake and this was dependent on protein synthesis. A maximum increase in both cystine uptake and intracellular GSH concentration was seen at 16 to 24 hours after the addition of arsenite. Similar results were obtained when cells were treated with other glutathione-depleting or oxidising agents such as DEM, BSO, BCNU, hydrogen peroxide and hyperoxia. The uptake rate of cystine is increased three fold when cells are exposed to H_2O_2. It seems likely that up-regulation of cystine transport is an important adaptive response to oxidant-related stress *in vivo*. Under normal conditions utilisation of circulating cysteine might be adequate for glutathione synthesis but during stress conditions the sulphur amino acid pool may have physiological importance. It is intended that future work on the cell lines described in this thesis will include the study of cystine uptake.

(b) Transport of the menadione-glutathione conjugate

Menadione is known to form a conjugate with glutathione and there is an expanding literature on the mechanisms of removal of glutathione S-conjugates from the cell. In particular the existence of a specific molecular membrane pump for the transport of glutathione S-conjugates and GSSG from the cell has now been established (Ishikawa, 1989). The glutathione S-conjugate carrier (which has been designated Gs-x) has been studied using plasma membrane vesicles from rat heart, rat liver and human erythrocytes (Ishikawa, 1989). This is an ATP-dependent process which is inhibited by vanadate suggesting that phosphorylation of the carrier protein takes place. The carrier exhibits broad substrate specificity towards different types of glutathione S-conjugates. It is important for the elimination of conjugated xenobiotics and endogenous glutathione conjugates such as leukotriene C4. Adriamycin, daunorubicin and verapamil are good substrates for the membrane pump that mediates multidrug resistance (P-glycoprotein) but do not interfere with the transport of glutathione S-conjugates. This is evidence that Gs-x is a separate entity from P-glycoprotein (Ishikawa, 1990). Glutathione S-conjugates are anions under physiological conditions and their negative charge appears to be important to the function of the carrier. The Gs-x carrier has a higher affinity for conjugates with long aliphatic chains rather than those with an aromatic ring. It probably exists in all organs. Kinetic data have revealed three functionally essential domains at the active

site of the protein. These are "P" (which undergoes phosphorylation), "G" (which recognises the GSH moiety) and "C" (that has affinity towards the hydrophobic moiety of glutathione S-conjugates).

The accumulation of glutathione conjugates may be deleterious to the cell. Several glutathione S-conjugates have been found to inhibit both GST and glutathione reductase. Therefore the export of conjugates is a critical part of xenobiotic metabolism and is particularly important in cases where conjugation results in bioactivation rather than detoxification. The menadione-glutathione conjugate, for example, takes part in redox-cycling and so is potentially cytotoxic. Other examples are dichlorethanes or dibromoethanes which are activated on glutathione conjugation to genotoxic electrophiles such as episulphonium ions. The GS-x protein has not yet been purified. Future work would include experiments to determine whether a menadione-glutathione conjugate is exported from the cell via the Gs-x pump, and whether the activity of this pump changes as cells acquire resistance to menadione.

(c) Putative oxidative stress-inducible stimulon in mammalian cells

The acquisition of menadione-resistance is associated with alterations in a variety of antioxidant defences. Their relative importance is uncertain but it is clear that menadione resistant cells recruit a combination of mechanisms to mitigate the consequences of oxidative stress. Gille *et al* (1988) isolated oxygen-resistant strains of *E. coli* that were capable of growth in 99% oxygen. The activities of Cu/Zn-SOD, Mn-SOD, glutathione peroxidase and catalase were found to be elevated 2.5-, 2.1-, 1.9- and 4-fold respectively. It was considered likely that this was due to a genetic alteration in a single regulatory gene with a role analogous to that of the bacterial transcription factor, OxyR. In the cell lines described in this thesis, several of the changes observed in the drug-resistant cells appear to be the same as those observed after transient oxidative stress in wild-type cells. Induction of GST-Pi, GSHPx and heme oxygenase was seen in wild-type cells after transient oxidative stress and are constitutively over-expressed in menadione-resistant cells. It is tempting to ascribe these changes to mutation of a gene that regulates the adaptive response to oxidants in mammalian cells. The transcription factor, NF-κB, appears in certain mammalian cells to have a role similar to that of OxyR in prokaryotes. The possibility that it might regulate the response to oxidative stress in menadione-sensitive and resistant cells was explored. However, there is no evidence to date that NF-κB activation is altered in menadione-resistant cells. Further work on the cell line would therefore

include a search for other transcription factors responsible for regulation and control of the mammalian response to oxidative stress.

(d) The role of heme oxygenase in antioxidant defence

In chapter 5 the expression of a variety of oxidative-stress inducible proteins is described. Heme oxygenase is known to be strongly induced by oxidative stress and appears to be constitutively overexpressed in menadione-resistant cells. Whether heme oxygenase plays an active role in antioxidant defence is disputed (Nath, 1994). Further work on the menadione-resistant cell lines should include measurement of heme oxygenase enzyme activity in resting and stressed states. Also, it should be possible to establish whether heme oxygenase plays a direct role in antioxidant defence by the use of tin protoporphyrin which competitively inhibits HO activity. The partial or complete reversal of menadione-resistance by this agent would be strong evidence for a direct role of HO in menadione-resistance.

CONCLUSION

Resistance to menadione and other oxidants in mammalian cells is due to a combination of many contributing factors. Intriguingly many of the changes observed in menadione-resistant cells appear to be the same as those observed after transient oxidative stress in wild-type cells. This suggests that immortalisation of an adaptive response has occurred. It is hoped that further characterisation of the cell lines described here will help elucidate the mammalian response to oxidants. This is an important field of research for over the last few years the damage caused by oxyradicals has been implicated in many pathological processes. The acquisition of resistance by malignant cells to chemotherapeutic agents and ionising radiation that generate oxyradicals is a major obstacle to improving cancer cure rates. A greater understanding of the mechanisms that control resistance to oxidative stress may eventually allow therapeutic modulation of this process and ultimately contribute to improved survival for some cancer patients.

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